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(54) Title: SYNTHETIC SIGNALLING MOLECULES

(57) Abstract

The invention relates to synthetic signalling molecules, which are based on sequences derived from primary signalling motifs such as immunoglobulin tyrosine receptor–based activation motifs (ITAMs). The use of such signalling molecules within chimeric receptor proteins allows one to tailor the level of intracellular signalling mediated by the chimeric receptor. Proteins containing, and nucleic acids encoding, such synthetic signalling molecules suitable for use in medicine, are described.

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SYNTHETIC SIGNALLING MOLECULES

The present invention relates to novel synthetic signalling molecules and to methods for their production. In particular, the invention relates to synthetic signalling molecules based on sequences derived from primary signalling motifs such as immunoglobulin tyrosine receptor-based activation motifs (ITAMs).

Activation of many effector cells is initiated by a binding event at the cell surface that leads to the activation of one or more intracellular secondary messenger systems. This binding event activates signalling cascades that cause effects downstream of the binding event and that promote the activation of various biological functions of the cell.

Chimeric receptors have been designed that utilise these natural activation mechanisms and which can induce certain physiological effects. Extracellular ligand binding groups have been transplanted onto intracellular signalling domains so that the activation of specific secondary messenger systems can be initiated by the binding of a ligand of choice. In this fashion, the activation of receptor-bearing cells has been achieved through targeting cells such as T-cells to those cells or to other cells that express antigenic ligands on their surface.

In a chimeric receptor, ligand recognition is provided by an extracellular binding region. Accordingly, when designing a chimeric receptor, a suitable extracellular binding region is chosen which binds specifically to a target ligand with high affinity. Binding of ligand to the chimeric receptor triggers a series of intracellular events leading to activation of the receptor-bearing cell. This activation is effected by the presence in the chimeric receptor molecule of an intracellular signalling domain. Activation of this domain may lead to a variety of biological effects caused by the cell, such as increased cellular proliferation, increased expression of cytokines with, for example, pro- or anti-inflammatory responses, stimulation of cytolytic activity, differentiation of other effector functions, antibody secretion, phagocytosis, tumour infiltration and/or increased cellular adhesion.

Different classes or types of intracellular signalling domain promote the activation of different secondary messenger systems, and it has been proposed previously that the appropriate choice of intracellular signalling domain in a chimeric receptor can influence the type of activation promoted by a particular binding event.

The relationship between the level of expression of a chimeric receptor protein and strength of intracellular signalling generated appears to vary between different systems.

The therapeutic potential of partly or wholly synthetic chimeric receptor systems is at present limited by the degree to which intracellular messenger systems may be activated. Studies are currently underway in many laboratories aimed at elucidating the individual contributions of each of the components of the signalling cascade in immune effector cell function. Chimeric receptors with truncated, minimal and replicated intracellular signalling domains containing one or more natural primary signalling motifs in their natural arrangements have been constructed for the purpose of addressing these issues (Irving, B. *et al.*, 1993, J. Exp. Med. 177:1093; Steeg, C. *et al.*, 1997, Eur. J. Immunol. 27:2233). Signalling molecules with substitutions in the region separating the primary signalling motifs have been constructed (Vely, F. *et al.*, 1997, Eur. J. Immunol 27:3010) for use in analysis of ZAP-70 binding to the ζ chain of the TCR. In addition, signalling from chimeric receptors with single amino acid substitutions in the first ITAM of ζ has been studied (Sunder-Plassmann, R. *et al.*, 1997, Eur. J. Immunol. 27:2001). However, to date, it has not proved possible to improve the efficiency of naturally-occurring signalling domains to any significant extent.

It would be of great value to be able to tailor the level of intracellular signalling and thus the level of effector cell activation to a required degree. It would also be extremely desirable to be able to activate a cell and induce its biological functions with greater efficacy than is presently possible. The present invention addresses these difficulties by providing novel synthetic signalling molecules.

Summary of the invention

According to the present invention there is provided a nucleic acid encoding a synthetic signalling molecule comprising two or more sequence blocks (SBs) encoding primary signalling motifs linked in an arrangement in which said primary signalling motifs do not occur naturally.

It has been found, surprisingly, that the combination of two or more primary signalling motifs, preferably different primary signalling motifs, in an arrangement that does not occur naturally, generates a functional synthetic signalling molecule that possesses potent biological properties. When such a synthetic signalling molecule is incorporated into an

immunoreceptor protein expressed in an effector cell, it confers on that protein the ability to initiate the activation of downstream intracellular signalling pathways that are naturally governed by phosphorylation events occurring within primary signalling motifs. The non-natural combination of primary signalling motifs in a chimeric receptor protein results in the modulation of the degree of actual activation mediated by the intracellular signalling domain. By altering the combination and/or number of signalling motifs within an intracellular signalling domain, it has been found, surprisingly, that the degree of cellular activation, and hence, the nature of certain biological processes (for example, cytokine release, induction of cell killing) resulting from the contact of a ligand with its extracellular ligand binding domain in a receptor, can be tailored as required.

By the term "primary signalling motif" is meant herein a sequence that is able to act in a stimulatory or inhibitory manner to elicit primary activation of an immune cell receptor complex (such as the TCR complex). Immunoreceptor tyrosine-based activation motifs (ITAMs) or immunoreceptor tyrosine-based inhibitory motifs (ITIMs) are examples of such primary signalling motifs. By "motif" is meant a specialised region or sequence within a larger protein or domain. Thus, for example, a signalling molecule or an intracellular signalling domain may naturally comprise more than one signalling motif.

ITAMs are highly conserved motifs which contain a tyrosine residue in two canonical YXX(L/I) consensus sequences, separated by between 6 and 8 intermediate residues. By the term "ITAM " is meant herein a sequence broadly conforming to the consensus sequence (D/E)XX**Y**XX(L/I/V)X_{6-8}**Y**XX(L/I/V) (the standard single letter amino acid code, where X represents any amino acid, is used throughout this application). The tyrosine residues (highlighted in bold in the above consensus sequence) are substrates for the Src family of tyrosine kinases. Phosphorylation of these tyrosine residues is a key early event in signal transduction and initiates signal propagation by facilitating the SH2-mediated binding of molecules from the Syk/Zap70 family of phosphotyrosine kinases and by recruiting phosphoinositide-related enzymes (for example, phospholipase C and phosphoinositide-3 kinase) and adaptor proteins (for example, Vav, Crk and Grb-2). This transduces the binding event into activation of several metabolic pathways.

Site-directed mutagenesis studies have been performed to ascertain the essential characteristics of the ITAM sequences (Letourneur, F and Klausner, R.D. (1992) Science 255, 79-82; Amigorena *et al* (1992), Science 256, 1808-1812) and synthetic peptides have been used to

identify molecules that may be involved in downstream propagation (Kimura *et al.*, J. Biol. Chem. 271(44): 27962).

Many immunoreceptors, including T cell and B cell antigen receptors and Fc receptors, comprise multiple subunits each of which is specialised in extracellular binding or in signal transduction. The intracellular domains of signalling molecules of, for example, the TCR, CD3 and FcγRIII share common stimulatory primary signalling motifs or ITAMS (Reth, M., 1989, Nature, 383-384). An ITAM is usually present as a single copy, for example, in the CD3γ, δ and ε chains. However, the TCR zeta and eta chains each contain three ITAMs which differ in sequence.

There is growing interest in the qualitatively different roles performed by individual ITAMs (Osman, N. *et al.*, 1996, Eur. J. Immunol. 26,1063) and also in the effects afforded by amplification of ITAM sequences (van Oers, N. *et al.*, 1998, J. Immunol., 160:163).

ITAMs for use in the present invention may be naturally derived. Suitable naturally-occurring ITAM sequences include those derived from, the zeta chain of the TCR (including the first (TCRζ1), second (TCRζ2) and third (TCRζ3) ITAMs of zeta), FcRγ (such as FcγRIIIγ or FcεRIγ), FcRβ (such as FcεRIβ), CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b, or CD66d. These ITAMS have been found to vary in their efficacy of signal transduction. Particularly preferred ITAMs are those derived from TCRζ1, TCRζ2, TCRζ3 and FcεRIγ.

By the term "ITIM" is meant herein a sequence broadly conforming to the consensus sequence (T/S)XXYXXL (Muta *et al.*, 1994, Nature 368:70-73). Phosphorylation at the highlighted tyrosine residue is thought to be required for association of an immunoreceptor containing this motif with a negative regulator of immunoreceptor function, such as SHP (also known as PTP1C, HCP, SH-PTP1) (Shultz *et al.*, 1993, Cell, 73: 1445-1454; Tsui *et al.*, 1993, Nature Genetics, 4, 124-129).

ITIMs may be included in synthetic signalling molecules encoded by the nucleic acids of the invention in order to impart a negative regulatory function. Chimeric receptor molecules containing one or more ITIMs would be of particular use, for example, to down-regulate the B cell antigen receptor (see Thomas, 1995, J. Exp. Med. 181: 1953-1956).

ITIMs for use in the present invention may be naturally derived. Suitable naturally-occurring ITIM sequences include those derived from FcγR (such as FcγRIIB), CD22, EPOR, IL-2βR and IL-3βR.

As will be clear to those of skill in the art, variants of naturally-occurring primary signalling motifs, resulting from randomly generated or site-directed mutagenesis, may also be suitable for use in the present invention. Of course, combinations of natural and variant motifs may be used so that the nucleic acid contains both natural and non-natural sequences.

Nucleic acids encoding two or more primary signalling motifs, may be combined in series, linked together as part of a polypeptide chain.

According to a further aspect of the invention, one or more secondary signalling motifs may be included in the synthetic signalling molecule in addition to the primary signalling motifs. By "secondary signalling motif" is meant a motif that imparts secondary or costimulatory signalling capacity to a molecule in T cells. Molecules which contain secondary signalling motifs include CD2, CD4, CD8, CD28 and CD154 (see Finney *et al.*, 1998, J. Immunol., 161: 2791-2797). Preferred motifs are those derived from CD28 and CD154, and variants thereof. Examples of secondary signalling motifs derived from CD28 and CD154 are a SB28 (GSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAGS) and SB29 (GSMIETYNQTSPRSAATGLPISMKGS), respectively (see also Figure 5). Most preferably, the secondary signalling motif is SB28.

The nucleic acid molecules of the invention are assembled from components that take the form of "sequence blocks", with each sequence block encoding a particular primary or secondary signalling motif as defined herein. These sequence blocks may conveniently be coupled together by linker regions, that are preferably of six or more base pairs in length. In a particularly suitable arrangement, part of the nucleic acid molecule that codes for the linker region forms part of a recognition site for a restriction enzyme. By cutting the nucleic acid molecule with the appropriate enzyme or enzymes, a section of nucleic acid is generated that possesses cohesive termini at each end and that may easily be ligated to other sequence blocks with a compatible cohesive end. This system facilitates the cloning of synthetic signalling sequences that contain primary or secondary signalling motifs joined in varied numbers and arrangements. This system is described in further detail in a co-pending International patent application filed by Celltech Therapeutics Limited entitled

"Combinatorial Method for Producing Nucleic Acids" (Ref PA450; P021407WO) filed on even date herewith. This method allows a library of molecules to be generated, each containing the same extracellular ligand binding domain, transmembrane domain and spacer domains, but with different forms of primary or secondary signalling motif, in a random or specified order in the synthetic signalling sequence.

In addition to forming part of a restriction endonuclease recognition site, the linker may also encode two (where the linker is 6 base pairs) or more (where the linker is more than 6 base pairs) amino acids. A particularly suitable linker encodes Gly-Ser. Figure 5 shows the amino acid sequence encoded by a number of synthetic sequence blocks. These sequences correspond to the sequences of various naturally-occurring ITAMs, but possess Gly-Ser linkers at each terminus. The coding sequence for the "Gly-Ser" doublet corresponds to the recognition site for the appropriate restriction enzyme.

Each sequence block may be generated by any appropriate conventional means, as will be clear to those of skill in the art. One particularly suitable method is to hybridise complementary single-stranded oligonucleotides to generate cohesive overhangs that may be ligated to each other. An alternative approach is to generate double-stranded nucleic acid molecules by PCR, and then to generate cohesive termini by digestion with the appropriate restriction enzymes.

As will be clear to the person of skill in the art, only one Gly-Ser linker is needed between each sequence block. Upon restriction digestion of the sequence blocks, part of a restriction site is formed at each end of the nucleic acid molecule, so that when the 5' end of one molecule is joined to the 3' end of another molecule, the resulting sequence contains a single Gly-Ser linker.

The amino acid sequences of preferred sequence blocks representing ITAMs include: SB1 (GSGQNQLYNELNLGRREEYDVLDKRRGRDPEMGS), SB2 (GSRKNPQEGLY-NELQKDKMAEAYSEIGMKGERGS), SB3 (GSRGKGHDGLYQGLSTATKDTY-DALHMQAGS), SB4 (GSYEKSDGVYTGLSTRNQETYETLKHEKPGS), SB4* (GSYEKSDGVYTGLSTRNQETYDTLKHEKPGS), SB4" (GLYEKSDGVYTGLGTR-NQETYETLKHEKPGS), SB5 (GSGNKVPEDRVYEELNIYSATYSELEDPGEM-SPGS), SB6 (GSKQTLLPNDQLYQPLKDREDDQYSHLQGNQLRGS), SB7 (GSALLRNDQVYQPLRDRDDAQYSHLGGNWARNKGS), SB8 (GSQNKERPPPVP-

NPDYEPIRKGQRDLYSGLNQRRIGS) SB9 (GSHVDNEYSQPPRNSRLSAYPAL-EGVLHRSGS), SB10 (GSPPRTCDDTVTYSALHKRQVGDYENVIPDFP-EDEGS), SB11 (GSEYEDENLYEGLNLDDCSMYEDISRGLQGTYQDVGS), SB12 (GSKAGM-EEDHTYEGLDIDQTATYEDIVTLRTGEVGS) and SB13 (GSPLPNPRTAASIYEELL-KHDTNIYCRMDHKAEVAGS) (See also Figure 5). Particularly preferred sequences are SB1, SB2, SB3, SB4, SB4" and SB4*.

The number of primary signalling motifs included in a synthetic signalling molecule has been found to affect the efficacy of the molecule in effecting downstream activation of intracellular messenger systems. Preferably, between two and ten primary signalling motifs are included. More preferably, between 2 and 5 primary signalling motifs are included.

The order in which the primary signalling motifs appear in the synthetic signalling molecule has also been found to affect the signalling efficacy of the molecules of the invention. For example, it is preferred to include at the N terminal end of a synthetic signalling molecule the second ITAM of TCRζ, for example SB2. Either the first ITAM of TCRζ, for example SB1, or the ITAM of FcεRIγ, for example SB4 (or SB4" or SB4*), is preferred at the second position, whilst at the third (C-terminal) position in the sequence, the third ITAM of TCRζ (for example SB3) is preferred. When inserted into the intracellular domain of a full-length protein sequence, the N terminus of the synthetic signalling molecule will be situated in a membrane-proximal position.

The order in which secondary signalling motifs appear in the synthetic signalling molecule has also been found to affect the signalling efficacy of the molecules of the invention. A preferable position for a secondary signalling motif is the N terminal side of the primary signalling motifs, i.e. in a membrane proximal position when the synthetic signalling molecule is inserted into the intracellular signalling domain of a full-length protein sequence.

According to a preferred aspect of the invention, there is provided a nucleic acid molecule encoding a synthetic signalling molecule that contains one or more of SB1 to SB13 inclusive. The encoded molecules have been found to possess enhanced biological function when compared to the function of primary signalling motifs in naturally-occurring proteins. A particularly preferred nucleic acid molecule encodes a synthetic signalling molecule

containing the coding sequence for SB4, or the coding sequence for SB4*, or the coding sequence for SB4".

According to a further aspect of the invention, there is provided a nucleic acid molecule encoding any one of the synthetic signalling regions listed in Table 1. Preferable nucleic acid molecules are those that encode proteins, which exhibit enhanced biological function when compared to the function of naturally-occurring proteins, for example, molecules that confer more effective cell killing or greater efficacy in releasing cytokines in response to antigenic challenge.

According to a further aspect of the invention there is provided a peptide or polypeptide synthetic signalling molecule encoded by a nucleic acid according to the above-described aspects of the invention. Particularly suitable combinations of primary and secondary signalling motifs are given in Table 1.

Synthetic signalling molecules may be constructed from ITAMs derived from TCRζ1, TCRζ2, TCRζ3, FcεRIγ, in the following preferred combinations: TCRζ2-TCRζ1-TCRζ3-TCRζ1; TCRζ2-FcεRIγ-TCRζ3; TCRζ2-FcεRIγ-TCRζ3-TCRζ2; TCRζ2-TCRζ1-TCRζ3-FcεRIγ; TCRζ2-TCRζ1-TCRζ3-TCRζ2; TCRζ2-TCRζ1-TCRζ3; TCRζ2-FcεRIγ-TCRζ3-TCRζ1; TCRζ1-TCRζ2-TCRζ3; and TCRζ2-TCRζ1 and optionally in each instance a secondary signalling motif (as defined herein) positioned N terminal to said signalling molecule. These molecules have been found to confer advantageous biological properties on a chimeric immunoreceptor when included in the intracellular domain.

Examples of particularly preferred synthetic signalling molecules are: SB2-SB1-SB3-SB1; SB2-SB4-SB3; SB2-SB4-SB3-SB2; SB2-SB1-SB3-SB4; SB2-SB4-SB3-SB29; SB2-SB1-SB3-SB2; SB2-SB1-SB3; SB2-SB4 -SB3-SB1; SB1-SB2-SB3; SB2-SB1; SB1-SB4"; and SB4*-SB2-SB4. Optionally, in each instance a secondary signalling motif (as defined herein) is positioned N terminal to said signalling molecule, for example, SB28.

According to a further aspect of the invention there is provided a chimeric receptor molecule comprising a synthetic signalling molecule encoded by a nucleic acid according to any one of the above-described aspects of the invention.

Where desired, the synthetic signalling molecule can be included in the intracellular signalling domain of a receptor. The intracellular signalling domain may be a naturally-

occurring polypeptide signalling sequence or may be partly or wholly synthetic. Examples of suitable naturally-occurring sequences include sequences derived from: the T cell receptor, such as all or part of the zeta, eta or epsilon chain; CD28; CD4; CD8; the gamma chain of an Fc receptor or signalling components from a cytokine receptor, such as the interleukin, TNF or interferon receptors; a colony stimulating factor receptor e.g. GMCSF, tyrosine kinase e.g. ZAP-70, fyn, lck, Itk and syk; and binding domains thereof; an adhesion molecule e.g. LFA-1 and LFA-2; B29; MB-1; CD3 delta; CD3 gamma; CD5; or CD2. As the skilled artisan will appreciate, amino acid mutations, deletions, insertions or substitutions may be made from natural sequences in order to modify the precise properties of the domains, in accordance with what is required for the chimeric receptor molecule.

These signalling motifs may be combined so as to allow the activation of a number of secondary messenger cascades through a single binding event at an extracellular ligand binding domain. As will be clear to the skilled artisan, combinations of intracellular signalling motifs can be on separate polypeptide chains or may be in series on a single polypeptide chain.

In order to distance the motifs appropriately from each other or from the transmembrane domain of the receptor, one or more spacer domains may be used. Spacer domains have been previously used in chimeric receptor design to link the domains to each other or to arrange the domains of the receptor in a desired conformation to optimise the binding or signalling potential of the molecule. The inclusion of such domains in the receptor may also facilitate the initial cloning steps when the nucleic acid elements encoding each domain of the protein are assembled.

Other components of the receptor may be included as appropriate for the function that is desired. In this respect, reference is made to two co-pending International patent applications, both entitled "Biological Product" (Refs. PA448, PA449) filed by Celltech Therapeutics Limited on even date herewith.

The extracellular domain of the chimeric receptor should exhibit specificity for a specific ligand or class of ligands, by virtue of inclusion in the chimeric protein of an extracellular ligand binding domain. As used herein, the term "extracellular ligand binding domain" is intended to refer to any oligo- or polypeptide that is capable of binding to a ligand. The term thus includes antibody binding domains, antibody hypervariable loops or CDRs,

receptor binding domains and other ligand binding domains, examples of which will be readily apparent to those of skill in the art.

The mechanism by which the binding of ligand to the extracellular ligand binding domain of the chimeric protein is transduced to the intracellular activation of the signalling domain is unclear. The mechanism most likely relies on some form of conformational change in the protein that exposes the primary or secondary signalling motifs to intracellular kinases, so facilitating their phosphorylation. Accordingly, when designing a chimeric receptor according to the invention, the choice of extracellular ligand binding domain will depend upon the type and number of ligands that define the surface of a target cell.

Preferably, the extracellular ligand binding domain is capable of interacting with a cell surface molecule. Parts of receptors associated with binding to cell surface-associated molecules and especially include an antibody variable domain (V_H or V_L), a T-cell receptor variable region domain (TCRα, TCRβ, TCRγ, TCRδ) or a CD8α, CD8β, CD11A, CD11B, CD11C, CD18, CD29, CD49A, CD49B, CD49C, CD49D, CD49E, CD49F, CD61, CD41 or CD51 chain. Of course, fragments of these domains or chains may be used where appropriate.

For example, the extracellular ligand binding domain may be chosen to recognise a cell surface marker ligand expressed on target cells associated with a disease state. Accordingly, surface marker ligands associated with viral, bacterial and parasitic infection, autoimmune disease and cancer cells are suitable. Examples of markers for cancer cells are the bombesin receptor expressed on lung tumour cells, CEA, PEM, CD33, Folate receptor, epithelial cell adhesion molecule (EPCAM) and *erb*-B2. Other molecules of choice are cell surface adhesion molecules, inflammatory cells present in auto-immune disease and T-cell receptors or antigens that give rise to autoimmunity. Further examples will be readily apparent to those of skill in the art.

The chimeric receptors may be designed so as to be bi- or multi-specific. Proteins which feature more than one extracellular ligand binding domain may, for example, recruit cellular immune effector cells such as T-cells, B-cells, NK-cells, macrophages, neutrophils, eosinophils, basophils or mast cells or components of the complement cascade. A particularly suitable combination of ligand specificities is anti-CD3 with anti-CD28, to specifically recruit and stimulate T-cells.

As will be clear to the skilled artisan, these combinations of extracellular ligand binding domains can be on separate polypeptide chains or may be in series on a single polypeptide chain.

It may also be desired for the extracellular ligand binding domains to be able to interact co-operatively with each other to form a ligand binding site. Particular examples include a V_H domain paired with a V_L domain, two or more TCRα, TCRβ, TCRγ and/or TCRδ domains, a CD8α or CD8β homo or heterodimer, CD18 paired with one or more of CD11a, b, or c, CD29 paired with one or more of CD49a, b, c, d, e or f and CD61 paired with CD41c and/or CD51. In this aspect of the invention, in binding to ligand, each extracellular ligand binding domain forms part of a ligand binding site and in doing so establishes a close spatial proximity of the chains which constitute the chimeric receptor.

In one aspect of the invention, the extracellular ligand binding domain may be chosen such that it interacts with one or more other extracellular ligand binding domains of other receptors of the same or different types. Indeed, naturally-occurring members of the MIRR family interact with multivalent antigens, and initiate their cellular response through a process that is dependent on aggregation of the receptors (Metzger, (1992), J. Immunol., 149:1477-1487).

In the same way, spacer domains may be incorporated into a chimeric receptor protein that either do, or do not associate with one another or with other proteins in the host cell membrane. By using non-associating spacer domains, constitutive association of the chimeric receptor proteins may be minimised to prevent their constitutive activation. Of course, the opposite effect may also be achieved if constitutive activation is desired. Either possibility may be achieved artificially by deleting, inserting, altering or otherwise modifying amino acids and naturally occurring sequences in the transmembrane and/or spacer domains which have sidechain residues that are capable of covalently or non-covalently interacting with the side chains of amino acids in other polypeptide chains. Particular examples of amino acids that can normally be predicted to promote association include cysteine residues, charged amino acids or amino acids such as serine or threonine within potential glycosylation sites.

The chimeric receptors of the invention also comprise a transmembrane region. In many cases, the transmembrane domain that is naturally linked to the extracellular or intracellular

portion of the chimeric receptor may be retained. However, it has been discovered that it is possible to change the biological properties of membrane-associated proteins simply by substituting the natural transmembrane domain for a transmembrane domain that is not naturally part of the molecule or which is not naturally fused to either of the extracellular or intracellular domains of the receptor. A synthetic transmembrane region may also be used. For the avoidance of doubt, the term "not naturally part" as used herein is intended to mean that the transmembrane domain does not naturally exist in association with the adjoining domains to which it is attached.

The biological properties which may be altered by substitution of the transmembrane region include the level of expression. Accordingly, the level of expression of chimeric receptor may be matched to the level of expression of the particular target ligand for the receptor, so that the optimal degree of receptor cross-linking, clustering and oligomerisation can be achieved. This allows optimisation of activation of the signalling cascade within the cell in which the chimeric receptor protein is expressed. In this respect, specific transmembrane domains may be chosen to optimise cross-linking of the protein with other components of the membrane. The degree to which the level of expression of the protein has been altered by replacing the transmembrane domain can be assessed by a number of methods, such as by fluorescence-activated cell sorting (FACS) or Western blotting using an antibody specific for the protein of interest, or by monitoring the level of cytokine release in response to cell-bound and/or soluble antigen.

Substitution of transmembrane region may also be used to change the sensitivity of the chimeric receptor protein for antigen. Furthermore, changing the transmembrane component also allows alteration of the ratio of cell surface-associated antigen to soluble antigen that is bound by a membrane-associated protein. Substitution of transmembrane region is described in detail in co-pending International patent applications filed by Celltech Therapeutics Ltd., entitled "Biological Product" (Ref PA 448) and "Method" (Ref PA 480) filed on even date herewith.

The transmembrane domain may be naturally-occurring, in which case the domain may be derived from any membrane protein other than the protein into which the domain is to be introduced. Of particular interest in this aspect of the invention are transmembrane domains derived from all or part of the alpha, beta or zeta chain of the T cell receptor, CD28, CD3epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80,

CD86, CD137, or CD154. The transmembrane domain of a cytokine receptor may also be used (for example an interleukin receptor, a TNF receptor, or an interferon receptor). It may also be derived from a colony stimulating factor receptor such as GMCSF receptor. Further examples will be clear to those of skill in the art.

Alternatively, the transmembrane component may be synthetic. Suitable synthetic transmembrane components will comprise predominantly hydrophobic amino acids such as leucine and valine. It has also been found preferable to include at each end of the transmembrane component the triplet phenylalanine, tryptophan, valine (FWV).

It is intended that chimeric molecules according to the present invention be applied in methods of therapy of mammalian, particularly human, patients. By using the specificity of the extracellular ligand binding domain to define the precise ligands or class of ligands that are capable of activating the receptor, the receptor may be tailored to generate a cellular response by the cell in which the receptor is expressed. Chimeric proteins generated by the present invention may be particularly useful in the treatment of diseases or disorders such as those described under the general headings of infectious diseases, e.g. HIV infection; inflammatory disease/autoimmunity e.g. rheumatoid arthritis, osteoarthritis, inflammatory bowel disease; cancer; allergic/atopic diseases e.g. asthma, eczema; congenital e.g. cystic fibrosis, sickle cell anaemia; dermatologic, e.g. psoriasis; neurologic, e.g. multiple sclerosis; transplants e.g. organ transplant rejection, graft-versus-host disease; metabolic/idiopathic disease e.g. diabetes.

For example, expression of the chimeric receptor on the surface of a T cell may initiate the activation of that cell upon binding of the extracellular domain to ligand on a target cell. The ensuing release of inflammatory mediators stimulated by activation of the signalling function of the receptor ensures the destruction of the target cell.

When a chimeric receptor produced according to the present invention is expressed in an effector cell of the immune system, binding to target will activate the effector cell; downstream effects of this activation may also result in the direct destruction of the target cell.

If the extracellular ligand binding domain of the chimeric protein shows specificity for a surface marker on an immune cell, effector cells may be attracted to the site of disease.

Accordingly, expression of the chimeric protein in a diseased cell will ensure its destruction.

Of course, more than one extracellular ligand binding domain may be included in the chimeric receptor protein or more than one type of chimeric receptor protein may be expressed in a single effector cell, so giving multiple specificity. Accordingly, binding of the chimeric receptor to its target may not only activate the effector cell itself, but may attract other immune effector cells to the site of disease. Once the immune system has been activated or recruited, the normal function of the immune system takes over and the target cell may thus be destroyed.

Chimeric receptor proteins that possess more than one extracellular ligand binding domain may, for example, recruit cellular immune effector cells such as T-cells, B-cells, NK-cells, macrophages, neutrophils, eosinophils, basophils or mast cells or components of the complement cascade. A particularly suitable combination of ligand specificities is anti-CD3 with anti-CD28, to specifically recruit and stimulate T-cells.

Examples of suitable effector cells for expression of the chimeric receptors of the invention include cells associated with the immune system such as lymphocytes e.g. cytotoxic T-lymphocytes, tumour infiltrating lymphocytes, natural killer cells, neutrophils, basophils or T-helper cells, dendritic cells, B-cells, haematopoietic stem cells, macrophages, monocytes or natural killer (NK) cells. Expression in cytotoxic T-lymphocytes is especially preferred.

Nucleic acids encoding the synthetic signalling molecules or chimeric receptors of the invention may be introduced into host cells, either by vector or any other suitable carrier or delivery system, and expressed in such host cells using the procedures generally described in International patent applications WO97/23613 and WO99/00494 and as described in the examples included herein.

As described above, the nucleic acids and/or polypeptides of the invention may be employed in the treatment of a number of different diseases or disorders occurring in mammalian subjects, and the invention thus extends to a method of treatment of such subjects. Such a method comprises administering to the subject an effective amount of a nucleic acid or polypeptide as described above. The exact amount to be administered will depend on the age and condition of the patient, the nature of the disease or disorder and the

rate of administration, but may be determined using conventional means, for example, by extrapolation of animal experiment derived data. In particular, in *ex vivo* therapies, the number of transfected effector cells required may be established by *ex vivo* transfection and re-introduction into an animal model at a range of effector cell numbers. Similarly, the quantity of nucleic acid required for *in vivo* use may be established in animals using a range of DNA concentrations.

According to a further aspect of the invention, there is provided a composition comprising a nucleic acid molecule, synthetic signalling sequence or chimeric receptor molecule according to any of the above described aspects of the invention, optionally in conjunction with a pharmaceutically-acceptable excipient.

If the composition is suitable for oral administration the formulation may contain, in addition to the active ingredient, additives such as: starch – for example, potato, maize or wheat starch or cellulose - or starch derivatives such as microcrystalline cellulose; silica; various sugars such as lactose; magnesium carbonate and/or calcium phosphate. It is desirable that, if the formulation is for oral administration it will be well tolerated by the patient's digestive system. To this end, it may be desirable to include in the formulation mucus formers and resins. It may also be desirable to improve tolerance by formulating the compositions in a capsule which is insoluble in the gastric juices. It may also be preferable to include the composition in a controlled release formulation.

According to a still further aspect of the invention there is provided a method of generating a synthetic signalling sequence comprising combining two or more different primary signalling motifs in an order in which they do not naturally occur. Secondary signalling motifs may also be included in the synthetic signalling sequence. The intention of this method is to generate a synthetic signalling sequence that exhibits increased potency over naturally-occurring signalling sequences, either in inducing cell killing, or effecting cytokine release in response to antigen challenge when said synthetic signalling sequence is expressed in an effector cell.

The invention will now be described in further detail with specific reference to chimeric receptor molecules generated by the combination of SBs in a pBLUESCRIPT-based system. It will be appreciated that variation may be made from these specific examples without departing from the scope of the invention.

All documents cited herein are incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic diagram showing system for assembly of SBs.

Figure 2: Cloning cassette for construction of chimeric receptors with synthetic signalling components.

Figure 3: Sequence of cloning cassette.

Figure 4: Oligonucleotide sequences for chimeric receptor construction.

Figure 5: Predicted amino acid sequence of SBs.

Figure 6: Level of expression and degree of activation of chimeric receptors containing synthetic signalling components by surface-bound antigen.

Figure 7: Activation of chimeric receptors containing synthetic signalling components by surface-bound antigen.

Figures 8-11: Activation of chimeric receptors containing synthetic signalling components by surface-bound antigen.

Figure 12: Cytokine production by human PBMC effectors expressing chimeric receptors.

Figure 13: Cytokines production by murine effectors expressing chimeric receptors.

Figure 14: *In vitro* cell killing.

Figure 15: *In vivo* immunotherapy.

EXAMPLES

These sequence blocks (SBs) are constructed by annealing two synthetic strands of DNA (oligos) so that the 5' end forms a Bcl I overhang and the 3' end forms a BamH I overhang. Both these restriction site overhangs are compatible with a BamH I site in the cloning vector. Insertion of the SB in the correct orientation destroys the 5' BamH I site and retains a 3' BamH I site, allowing subsequent 3' insertion of SB(s). Insertion of

the SB in the wrong orientation retains a 5 ' BamHI site and generates a 3' stop codon (Figure 1). In this Example the SBs have 5' and 3' phosphate groups and the BamH I site in the construction vector (Figure 2) has its phosphate groups removed to prevent vector re-ligation in the absence of insert ligation.

Example 1 :Construction of the cloning vector, pHMF393

To facilitate construction of chimeric receptors with different binding, extracellular spacer, transmembrane and signalling components, a cloning cassette system was devised in pBluescript SK+ (Stratagene). This is a modification of our cassette system described in International Patent Specification No. WO 97/23613.

This new cassette system is shown in Figure 2. The binding component has 5' Not I and Hind III restriction sites and a 3' Spe I restriction site. The extracellular spacer has a 5' Spe I site (Thr, Ser) and a 3' Nar I site (Gly, Ala). The transmembrane component has a 5' Nar I site (Gly, Ala) and 3' Mlu I (Thr, Arg) and BamHI sites (Gly, Ser). The signalling component may be cloned into the BamHI site. Following this BamH I site there is a stop codon for transcription termination and there is a EcoRI site 3' of this for subsequent rescue of whole constructs.

To generate this cassette, a 200bp fragment was PCR assembled using oligos:- S0146, A6081, A6082 and A6083 (Figure 4). This fragment starts with a SpeI site and consists of the extracellular spacer h.CD28, the human CD28 transmembrane region, a stop codon and finishes with an EcoRI site (see Figure 3). This PCR fragment was then restricted with SpeI and EcoRI and substituted for the same fragment in our previously described cloning cassette system to join the binding component (International Patent No. WO 97/23613 Figure 2).

Example 2: Construction of Chimeric receptors with full length natural signalling regions

These chimeric receptors will act as control chimeric receptors against which function of synthetic chimeric receptors will be compared. These new chimeric receptors with natural signalling regions include an optimised spacer component and transmembrane component as described in Patent application (PA448/TM); PCT/GB96/03209 and Finney *et al* : J.Immunol. 161, 2791-2797,1998.

a) P67scFv/h.CD28/CD28Tm/FcRγ chimeric receptor

This construct was generated from the cloning vector, pHMF393 described above. The FcRγ intracellular component was PCR cloned with oligos A9515 and A9516 (Figure 4) from human Leukocyte cDNA (Clontech) and cloned into the BamHI site of the described cassette (Figure 1).

The binding component, P67 single chain Fv (scFv) with specificity for CD33 and CD33 on HL60 cells, consists of a human antibody leader sequence and the variable component of the light chain of the engineered human antibody linked via a $(Gly_4Ser)_5$ linker to the variable component of the heavy chain of the engineered human antibody. This binding component is described in WO97/23613. The extracellular spacer component h.CD28, consists of residues 234 to 243 of human IgG1 hinge and residues 118 to 134 of human CD28. The transmembrane component consists of residues 135 to 161 of human CD28 (A. Aruffo & B. Seed 1987 PNAS 84 8573-8577). The intracellular component consists of residues 27 to 68 of the γ chain of human FcεR1 (Kuster *et al* (1989) J. Biol. Chem. 255, 6448-6452)

b) P67scFv/h.CD28/CD28Tm/TCRζ chimeric receptor

The TCRζ intracellular component was PCR cloned with oligos C3208 and C3209 (Figure 4) from previously described chimeric receptor, P67scFV/G1/Zeta (PCT/GB96/03209) on a Mlu I to EcoRI fragment and cloned into the Mlu I and EcoR I site of the described cassette (Figure 1).

The binding component, P67 single chain Fv (scFv) with specificity for CD33 and CD33 on HL60 cells, consists of a human antibody leader sequence and the variable component of the light chain of the engineered human antibody linked via a $(Gly_4Ser)_5$ linker to the variable component of the heavy chain of the engineered human antibody. This binding component is described in WO 97/23613. The extracellular spacer component h.CD28, consists of residues 234 to 243 of human IgG1 hinge and residues 118 to 134 of human CD28. The transmembrane component consists of residues 135 to 161 of human CD28 (A. Aruffo & B. Seed 1987 PNAS 84 8573-8577). The intracellular component consists of residues 31 to 142 of the ζ chain

of human TCRζ (Weissman *et al* : PNAS 85 ,9709-9713, 1988. Moingeon *et al.*, Eur. J. Immunol. 20, 1741-1745, 1990).

This product has been found to be particularly useful in a biological context in the treatment of cancer and forms a further feature of the present invention.

c) scFv/G1/zeta chimeric receptors

Two chimeric receptors were constructed as described in International patent applciation WO97/23613, and used as controls for *in vitro* and *in vivo* functional analysis (see Figures 14 and 15). These comprised the single chain Fvs, p67 (that demonstrates specificity for the CD33 antigen) and AB57 (which demonstrates specificity for CEA).

Example 3:The construction of sequence blocks (SBs)

Each sequence block was generated by annealing two oligos such that they have single-stranded overhangs forming half a Bcl I site at the 5' end and half a BamH I site at the 3' end. Oligos were annealed at a concentration of 1pmole/µl in a buffer consisting of :- 25mM NaCl, 12.5 mM Tris-HCl, 2.5mM $MgCl_2$, 0.25mM DTE, pH7.5 by heating in a boiling water bath for 5 minutes and then allowing the bath to cool slowly to room temperature.

The predicted amino acid sequences of these Examples of SBs are shown in Figure 5

a) SB1

This sequence is based on the first ITAM of human TCRζ and was constructed by annealing oligos A8816 and A8817 (Figure 4). Both these oligos have a 5' phosphate group.

b) SB2

This sequence is based on the second ITAM of human TCR ζ and was constructed by annealing oligos A8814 and A8815 (Figure 4). Both these oligos have a 5' phosphate group.

c) SB3

This sequence is based on the third ITAM of human TCR ζ and was constructed by annealing oligos A8812 and A8813 (Figure 4). Both these oligos have a 5' phosphate group.

d) SB4

This sequence is based on the ITAM of the γ chain of human FcεR1 and was constructed by annealing oligos A8810 and A8811 (Figure 4). Both these oligos have a 5' phosphate group.

e) SB4*

This sequence was originally generated in error by mis-annealment of the above oligos but was subsequently made by annealing oligos A8810B and A8811B (Figure 4). Both these oligos have a 5' phosphate group.

f) SB5

This sequence is based on the ITAM of the β chain of human FcεR1 and was constructed by annealing oligos A9000 and A9001 (Figure 4). Both these oligos have a 5' phosphate group.

g) SB6

This sequence is based on the ITAM of the γ chain of human CD3 and was constructed by annealing oligos A9002 and A9003 (Figure 4). Both these oligos have a 5' phosphate group.

h) SB7

This sequence is based on the ITAM of the δ chain of human CD3 and was constructed by annealing oligos A9004 and A9005 (Figure 4). Both these oligos have a 5' phosphate group.

i) SB8

This sequence is based on the ITAM of the ε chain of human CD3 and was constructed by annealing oligos A9006 and A9007 (Figure 4). Both these oligos have a 5' phosphate group.

j) SB9

This sequence is based on the ITAM of human CD5 and was constructed by annealing oligos A9008 and A9009 (Figure 4). Both these oligos have a 5' phosphate group.

k) SB10

This sequence is based on the ITAM of human CD22 and was constructed by annealing oligos A9010 and A9011 (Figure 4). Both these oligos have a 5' phosphate group.

l) SB11

This sequence is based on the ITAM of human CD79a and was constructed by annealing oligos A9012 and A9013 (Figure 4). Both these oligos have a 5' phosphate group.

m) SB12

This sequence is based on the ITAM of human CD79b and was constructed by annealing oligos A9014 and A9015 (Figure 4). Both these oligos have a 5' phosphate group.

n) SB13

This sequence is based on the ITAM of human CD66d and was constructed by annealing oligos A9016 and A9017 (Figure 4). Both these oligos have a 5' phosphate group.

o) SB28

This sequence is based on the costimulation motif of human CD28 and was constructed by annealing oligos A9018 and A9019 (Figure 4). Both these oligos have a 5' phosphate group.

p) SB29

This sequence is based on the costimulation motif of human CD154 and was constructed by annealing oligos A9020 and A9021 (Figure 4). Both these oligos have a 5' phosphate group.

Example 4: Construction of chimeric receptors with synthetic signalling regions by sequential addition

The vector, pHMF393 (described in Example 1) was digested with the restriction enzyme BamH I under the manufacturer's recommended conditions (New England Biolabs) and then treated with alkaline phosphatase under the manufacturer's recommended conditions (Boehringer Mannheim) for 10 minutes at 37°C. The linearised vector fragment was gel eluted from a 1% agarose gel and purified using a QIAquick as described by QIAGEN Ltd. Approximately 1 ng of vector fragment was ligated to approximately 1 pmole of SB or mixture of SBs using T4 ligase and ATP under the manufacturer's recommended conditions (New England Biolabs) in a 10 µl reaction. 2 µl of the ligation reaction was transformed into XL-1 blue competent E.coli as recommended by the supplier (Stratagene)and plated onto L-broth / Ampicillin plates. Ampicillin resistant colonies were screened for the presence of inserted SBs by PCR (PCR protocols, Innis *et al*, 1990, Academic Press Inc.) using oligos corresponding to regions 5' and 3' to the BamH I site in the vector. Positive colonies were picked and grown up in L-broth containing Ampicillin, and plasmid DNA prepared using QIAprep spin columns as described by QIAGEN Ltd. Correct orientation of the inserted SB was established by digesting the DNA with BamH I and an enzyme within the Vector (Nar I) under the manufacturer's recommended conditions (New England Biolabs).

Correct plasmids were then digested with BamH I, treated with alkaline phosphatase, gel purified and ligated to an SB or mixture of SBs as described above to insert a second SB. Colonies were screened again in the same way to find vectors with a second SB in the correct orientation. These vectors were then put through further rounds of digestion, purification, ligation and screening, as desired, to generate the required number of SBs in the correct orientation in a vector.

Specific Example: (see Table 1)

P67/h.CD28/CD28TM/SB2.SB1.SB3.SB1 (pHMF369) was constructed by the following steps of sequential addition:-

ligation of vector pHMF393 to SB2 to generate pHMF403

ligation of vector pHMF403 to SB1 to generate pHMF410

ligation of vector pHMF410 to SB3 to generate pHMF432

ligation of vector pHMF432 to SB1 to generate pHMF469.

Example 5: Construction of chimeric receptors with synthetic signalling regions by multiple addition

Construction of synthetic signalling regions with random combinations of SBs by adding mixtures of more than one SB at a time was done by the following methods:-

Experimental procedures are carried out under the recommended conditions as described in Example 3 except where specifically stated.

a) SBs were ligated to each other in the absence of vector and then digested with both Bcl I and BamH I to cut SBs ligated to each other in incorrect orientations. If a specific number of SBs were required, then the desired size fragments were gel eluted and purified; if not, the ligated SB fragments were purified from the restriction enzymes using QIAquick as described by QIAGEN Ltd. and then ligated to the digested, phosphatased and purified vector. Ampicillin-resistant colonies were then generated and screened to select vectors with more than one SB in the correct orientation in a vector.

b) SBs were ligated to the digested, phosphatased and purified vector and then digested with both Bcl I and BamH I to cut SBs ligated to each other and to the vector in incorrect orientations. Fragments larger than the unligated vector were gel purified and then ligated to recircularise. Ampicillin-resistant colonies were then generated and screened to select vectors with more than one SB in the correct orientation in a vector.

c) SBs were ligated to the digested, phosphatased and purified vector at a ratio of SB to vector that favoured the insertion of multiple SBs; Ampicillin resistant colonies were then generated and screened to select vectors with more than one SB in the correct orientation in a vector.

It was found that for maximum efficiency it was desirable to use method c and establish a titration of SB for each vector. A destimulation of colony number from vector only controls was a good indication of insertion of multiple SBs. It was also found to be more efficient to perform two rounds of ligation and screening for correct insertion of a low number of SBs rather than one round of ligation and screening for many SBs in the correct orientation. Statistically, adding 1 SB, 50% should be in the correct orientation; adding 2 SBs, 25% should be in the correct orientation but when 4SBs are added at one time only 6% of vectors would have all 4 SBs in the desired orientation.

In the case of multiple rounds of multiple insertion, mixtures of both vector and SBs were ligated to each other to increase diversity of the library produced.

Specific Examples: (see table 1)

P67/h.CD28/CD28TM/SB11.SB5.SB10.SB9 (pHMF537), P67/h.CD28/CD28TM/SB4.SB7.SB10 (pHMF538), P67/h.CD28/CD28TM/SB4.SB3 (pHMF539) and P67/h.CD28/CD28TM/SB4.SB1 (pHMF540) were constructed by ligating a mixture of linearised and phophatased vectors already containing one SB :- pHMF 403,404,405, 406, 515 and 516 to a mixture of SBs :- SB1,SB2,SB3,SB4,SB4*,SB5,SB6,SB7,SB8,SB9,SB10,SB11,SB12,SB13 and SB28.

P67/h.CD28/CD28TM/SB2.SB1.SB1 (pHMF529), P67/h.CD28/CD28TM/SB2.SB4.SB5 (pHMF530), P67/h.CD28/CD28TM/SB11.SB4*.SB3 (pHMF531) and P67/h.CD28/CD28TM/SB11.SB4*.SB10* (pHMF532) were constructed by ligating a mixture of linearised and phophatased vectors already containing two SBs :-pHMF 408,410,412, 518, 519, 520, 521 and 522 to a mixture of SBs :- SB1,SB2,SB3,SB4,SB4*,SB5,SB6,SB7,SB8,SB9,SB10,SB11,SB12,SB13 and SB28.

Example 6 : Cloning of full length CD28 into chimeric receptors with synthetic signalling components

Full length CD28 was cloned into the membrane proximal position of synthetic signalling components to provide increased co-stimulation. Residues residues 162 to 202 comprising the intracellular component of human CD28 (Aruffo & Seed : PNAS 84, 8573-8577) was PCR cloned using oligos D6996 and D6997 from a previously described chimeric receptor, P67scFV/ h.28 / CD28 (PCT/GB96/03209) on a Mlu I fragment and cloned into the Mlu I site upstream of the BamH I site of the cassette (Figure 2) into which synthetic signalling components have been built.

Example 7: Analysis of receptors

a) Construction of expression plasmids

The chimeric receptor constructs were subcloned from pBluescript KS+ into the expression vector pEE6hCMV.ne (C.R. Bebbington (1991), Methods 2, 136-145) on a HindIII to EcoRI restriction fragment. The expression vector with no chimeric receptor genes is used as a negative control in subsequent experiments.

b) Stable transfection into Jurkat E6.1 cells

The expression plasmids were linearised and transfected into Jurkat E6.1 cells (ECACC) by electroporation using a Bio-rad Gene Pulser. 10μg of DNA per 2.5 $X10^6$ cells were given two pulses of 1000V, 3μF in 1ml PBS. Cells were left to recover overnight in non-selective media before being selected and cultured in media supplemented with the antibiotic G418 (Sigma) at 1.5mg/ml. After approximately four weeks cells were ready for analysis.

c) FACS analysis of surface expression

Approximately $5X10^5$ Jurkat cells were stained with 1μg/ml FITC labelled antigen, CD33. Fluorescence was analysed by a FACScan cytometer (Becton Dickinson).

d) IL-2 production analysis of function

$2X10^5$ cells were incubated at 37°C/8% CO_2 for 20 hours in 96 well plates with HL60 target cells at an effector : target ratio of 1:1. Cell supernatants were then harvested and assayed for human IL-2 (R & D Systems Quantikine kit).

e) Functional analysis in primary cells

Human effector cells

Human PBMC blasts were prepared from donor blood by separation on Ficol followed by culture for five days in RPMI media with 10% FCS containing PHA at 5μg/ml and recombinant human IL-2 at 100U/ml.

Mouse effector cells

Spleens were recovered from BALB/c mice, and single cell suspensions prepared by passsage through nylon mesh. Red cells were lysed using the standard NCK lysing buffer; the remaining white cell fraction was cultured in DMEM with 10% FCS containing Con A at 2μg/ml and recombinant mouse IL-2 at 100U/ml.

Expression of chimeric receptors in primary cell blasts

Expression of chimeric receptors in both mouse and human primary effector cells was achieved by recombinant adenovirus according to the Adeno-quest protocol (Quantum Biotechnologies Inc.). In general, cDNA encoding the chimeric receptor was cloned into a transfer vector at a site flanked by a recombination sequence. This was then co-transfected into QBI-293A cells with part of the Ad5 viral genome so that the product of *in-vivo* recombination yielded recombinant infectious viruses which were replication incompetent. Following plaque purification and amplification steps, recombinant adenovirus was used at MOIs of 100-1000 to infect either mouse or human effector cells. Inclusion of DEAE dextran at 2μg/ml and sodium butyrate at 1.5mM in infection cultures was found to enhance expression of chimeric receptors. Infection with recombinant virus was carried out on day five of culture; effector cell populations expressing chimeric receptors were used on the day following infection. Cells were harvested and washed extensively before use as effectors in *in-vitro* assays and *in-vivo* in animal models.

Tumour target cells

A mouse NS0 cell line stably expressing the human CD33 antigen on its surface, under G418 selection, was used as a tumour target in *in-vitro* and *in-vivo* efficacy models. In certain assays, NS0 cells transfected with the same vector, but without the human CD33 gene insert, were used as control target cells.

In vitro efficacy: specific cytokine production

The ability of chimeric receptors to mediate specific cytokine production, when expressed in human PBMC blasts or mouse spleen cell blasts as effector cells and challenged with target cells, was assessed. Effectors and target cells were co-cultured for 20 hours at E:T ratio of 1:1. Samples of supernatants, containing secreted cytokines, were assayed by specific ELISA (RandD Systems). The range of cytokines produced included TNFα, TNFβ, IFNγ, GM-CSF, IL-2, IL-4 and IL-6.

In-vitro efficacy: cell killing

The ability of chimeric receptors with synthetic signalling regions, when expressed in primary effector cell blasts, to mediate specific killing of tumour target cells was tested in an *in-vitro* europium chelate cell killing assay. Mouse spleen effector cells were used as effectors, and NSO cells expressing human CD33 antigen were used as target cells. $3x10^6$ target cells were labelled with BATDA at 10μM for 20 minutes at 37°C. After extensive washing, 5,000 labelled target cells were incubated with effector cells expressing chimeric receptors at effector to target ratios (E:T) from 50:1 to 1:1 for up to four hours at 37°C. At harvest, cells were centrifuged, and samples of supernatant containing chelate, which had been released from target cells, were transferred to europium solution. Fluorescence was measured in a time resolved fluorometer with a delay of 1000μs, an integration time of 1000μs and with 100 flashes and 10ms between each flash. The specific chelate release following incubation of target cells with effector cells expressing chimeric receptors was compared to release caused by incubation of target cells with digitonin.

In-vivo efficacy: immunotherapy

$1x10^6$ human PBMC blasts expressing chimeric receptors were mixed with $1x10^6$ NS0 cells expressing CD33 antigen immediately prior to injection sub-cutaneously into nude mice.

Example 8 : Results

a) The chimeric receptors claimed in this invention are listed in Table 1. Figures 6 to 11 demonstrate signalling function of synthetic signalling components in relation to naturally-occurring signalling components when expressed in Jurkat. Function is demonstrated by specific cytokine production in response to cell bound antigen.

5

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Table I

pBluescript					EE6hC MVNE / Jurkat line	NOTES
	Binding	Spacer	Trans-membrane	Signalling Region		
pHMF403	hP67scFv	h.CD28	CD28	SB1	pHMF/ J.434	
404	hP67scFv	h.CD28	CD28	SB2	435	
405	hP67scFv	h.CD28	CD28	SB4	436	
406	hP67scFv	h.CD28	CD28	SB3	437	
407	hP67scFv	h.CD28	CD28	SB1.SB1	438	
408	hP67scFv	h.CD28	CD28	SB1.SB2	439	
409	hP67scFv	h.CD28	CD28	SB1.SB3	440	
410	hP67scFv	h.CD28	CD28	SB2.SB1	441	
411	hP67scFv	h.CD28	CD28	SB2.SB3	442	
412	hP67scFv	h.CD28	CD28	SB2.SB4	443	
427	hP67scFv	h.CD28	CD28	SB1.SB4	444	
428	hP67scFv	h.CD28	CD28	SB2.SB4*	445	1
429	hP67scFv	h.CD28	CD28	SB4.SB4*	446	1
430	hP67scFv	h.CD28	CD28	SB3.SB4*	447	1
431	hP67scFv	h.CD28	CD28	SB1.SB2.SB3	448	
432	hP67scFv	h.CD28	CD28	SB2.SB1.SB3	449	
433	hP67scFv	h.CD28	CD28	SB2.SB4.SB3	500	
469	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB1*	475	2
470	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB2	476	
471	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB3	477	
472	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB4	478	
473	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB1	479	3
474	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB2	480	3

501	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB28	507	3
502	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB1*.SB28		
503	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB2.SB28	508	
504	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB29	509	
505	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB1*.SB29		
506	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB2.SB29	510	3
511	hP67scFv	h.CD28	TM24	SB2.SB1.SB3.SB1*	512	2
513	hP67scFv	h.CD28	CD28	SB2.SB1.SB28	514	
515	hP67scFv	h.CD28	CD28	SB11	523	
516	hP67scFv	h.CD28	CD28	SB28		
517	hP67scFv	h.CD28	CD28	SB3'.SB4*		1,4
518	hP67scFv	h.CD28	CD28	SB11.SB4*	524	1
519	hP67scFv	h.CD28	CD28	SB3.SB3	525	
521	hP67scFv	h.CD28	CD28	SB4'*.SB1	527	1,5
522	hP67scFv	h.CD28	CD28	SB4*.SB3	528	1
529	hP67scFv	h.CD28	CD28	SB2.SB1.SB1	533	
530	hP67scFv	h.CD28	CD28	SB2.SB4.SB5	534	
531	hP67scFv	h.CD28	CD28	SB11.SB4*.SB3	535	1
532	hP67scFv	h.CD28	CD28	SB11.SB4*.SB10*	536	1,6
537	hP67scFv	h.CD28	CD28	SB11.SB5.SB10.SB9	559	
538	hP67scFv	h.CD28	CD28	SB4.SB7.SB10	560	
539	hP67scFv	h.CD28	CD28	SB4.SB3		
540	hP67scFv	h.CD28	CD28	SB4.SB1		
541	hP67scFv	h.CD28	CD28	SB1.SB3		
542	hP67scFv	h.CD28	CD28	SB28.SB1	561	
543	hP67scFv	h.CD28	CD28	SB11.SB7	562	
544	hP67scFv	h.CD28	CD28	SB3.SB13	563	
545	hP67scFv	h.CD28	CD28	SB28.SB4*	564	1
547	hP67scFv	h.CD28	CD28	SB2.SB1.SB2	565	
549	hP67scFv	h.CD28	CD28	SB4*.SB3.SB3	567	1
550	hP67scFv	h.CD28	CD28	SB1.SB2.SB9	568	
551	hP67scFv	h.CD28	CD28	SB1.SB2.SB12	569	
552	hP67scFv	h.CD28	CD28	SB4*'.SB1.SB1	570	1,5
553	hP67scFv	h.CD28	CD28	SB3.SB3.SB4*	571	1
554	hP67scFv	h.CD28	CD28	SB4*.SB1.SB12	572	1
555	hP67scFv	h.CD28	CD28	SB2.SB4.SB28	573	
556	hP67scFv	h.CD28	CD28	SB3.SB3.SB7	574	
557	hP67scFv	h.CD28	CD28	SB11.SB4*.SB10	575	1
558	hP67scFv	h.CD28	CD28	SB1.SB4*.SB3	576	1
577	hP67scFv	h.CD28	CD28	SB28.SB4*.SB2	589	1
578	hP67scFv	h.CD28	CD28	SB28.SB1.SB3	590	
579	hP67scFv	h.CD28	CD28	SB28.SB4*.SB4*	591	1
580	hP67scFv	h.CD28	CD28	SB28.SB1.SB28	592	
581	hP67scFv	h.CD28	CD28	SB28.SB1.SB2	593	
582	hP67scFv	h.CD28	CD28	SB28.SB1.SB13	594	
583	hP67scFv	h.CD28	CD28	SB28.SB3*	595	
584	hP67scFv	h.CD28	CD28	SB28.SB13	596	
585	hP67scFv	h.CD28	CD28	SB28.SB10	597	

586	hP67scFv	h.CD28	CD28	SB28.SB4*.SB2.SB4	598	1
587	hP67scFv	h.CD28	CD28	SB28.SB1.SB4"	599	7
608	hP67scFv	h.CD28	CD28	SB28.SB2	612	
609	hP67scFv	h.CD28	CD28	SB11.SB2	613	
610	hP67scFv	h.CD28	CD28	SB28.SB2.SB1	614	
611	hP67scFv	h.CD28	CD28	SB28.SB2.SB4	615	

NOTES TO TABLE 1

1) SB4* is a single amino acid different from SB4, initially generated by mis-annealment of oligos but subsequently deliberately generated by annealing oligos A8810B and A8811B (see Figure 4) due to enhanced activity.

SB4* :- GSYEKSDGVYTGLSTRNQETYDTLKHEKPGS

2) SB1* is a truncated version of SB1 generated by a recombination event during cloning.

SB1* :- GSGQNQLYNELNLGRREEYDVLAK

3) R to G change at the 5' end of SB3

4) A to T change at the 3' end of SB3

5) K to R change at the 5' end of SB4*

6) SB10* is a truncated ,version of SB10 with an altered 3' end.

SB10* :- GSPPRTCDDTVTYSALHKRQVGDYENVIPER

7) S to L change at the 5' end of SB4 and a S to G change in the middle of SB4.

SB4" :- GLYEKSDGVYTGLGTRNQETYETLKHEKPGS

b) *In vitro* efficacy: cytokine production

Figures 12 and 13 show that the levels of cytokines produced by effector cells expressing the chimeric receptor P67/h.CD28/CD28TM/SB28.SB4*.SB2.SB4 were significantly

higher than the amounts produced by effector cells expressing the standard chimeric receptor, with a full length zeta chain signalling region, on challenge with target cells expressing the specific antigen.

Effector cells expressing the chimeric receptor P67/h.CD28/CD28TM/SB28.SB4*.SB2.SB4 did not produce significant levels of cytokines constitutively or on challenge with target cells which did not express the specific antigen CD33.

c) *In vitro* efficacy: cell killing

No specific europium chelate release was seen from target cells that had been incubated with mouse spleen effector cells expressing a chimeric receptor with a full length zeta chain signalling region, but with an irrelevant specificity (A5B7 anti-carcinoembryonic antigen CEA). The chimeric receptor P67/h.CD28/CD28TM/SB28.SB4*.SB2.SB4 when expressed on mouse spleen cell blasts mediated significantly greater specific target cell killing than a chimeric receptor with a signalling region derived from the standard T cell receptor zeta chain (see Figure 14).

d) *In vivo* efficacy: immunotherapy

Tumours became palpable in control groups by around day 10. Animals that had received human PBMC blasts expressing a chimeric receptor with an irrelevant specificity (A5B7 anti-carcinoembryonic antigen CEA) developed tumours and did not survive beyond day 29. At an E:T ratio of 1:1, human PBMC blasts expressing the standard chimeric receptor, with a signalling region derived from the T cell receptor zeta chain, did not significantly prolong survival over the control group (see Figure 15). In contrast, all ten animals that had received human PBMC blasts expressing the chimeric receptor P67/h.CD28/CD28TM/SB28.SB4*.SB2.SB4 premixed with NS0 tumour target cells expressing CD33 did not go on to develop tumours.

CLAIMS

1. A nucleic acid encoding a synthetic signalling molecule comprising two or more sequence blocks (SBs) encoding primary signalling motifs linked in an arrangement in which said primary signalling motifs do not occur naturally.

2. A nucleic acid according to claim 1, wherein said primary signalling motifs are not the same primary signalling motifs.

3. A nucleic acid molecule according to claim 1 or claim 2, wherein each SB is connected to another SB by a linker containing six or more base pairs.

4. A nucleic acid molecule according to claim 3, wherein said linker encodes the sequence Gly-Ser.

5. A nucleic acid molecule according to any one of the preceding claims, wherein each primary signalling motif is an immunoreceptor tyrosine-based activation motif (ITAM).

6. A nucleic acid molecule according to claim 5, wherein each ITAM is derived from TCRζ1, TCRζ2, TCRζ3, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b or CD66d.

7. A nucleic acid molecule according to any one of claims 1-5, wherein each primary signalling motif is selected from sequences SB1, SB2, SB3, SB4, SB4*, SB4", SB5, SB6, SB7, SB8, SB9, SB10, SB11, SB12 and SB13.

8. A nucleic acid molecule according to claim 7, wherein one of said primary signalling motifs is SB1, SB2, SB3, SB4, SB4*, or SB4".

9. A nucleic acid molecule according to any one of claims 5-8, wherein the N-terminal primary signalling motif in the encoded synthetic signalling sequence is either the second ITAM of TCR ζ or is SB2.

10. A nucleic acid molecule according to any one of claims 5-9, wherein a second primary signalling motif in the encoded synthetic signalling sequence is either the first ITAM of TCR ζ, the ITAM of the γ chain of FcεRI, SB1, SB4 or SB4*, or SB4".

11. A nucleic acid molecule according to any one of claims 5-10, wherein a third primary signalling motif in the encoded synthetic signalling sequence is either the third ITAM of TCR ζ or is SB3.

12. A nucleic acid molecule encoding a synthetic signalling sequence which encodes one or more of SB1 to SB13 in Figure 5.

13. A nucleic acid molecule according to claim 12, wherein said synthetic signalling sequence comprises the coding sequence for SB4*.

14. A nucleic acid molecule according to claim 12, wherein said synthetic signalling sequence comprises the coding sequence for SB4".

15. A nucleic acid molecule according to any one of claims 1 to 4, wherein each primary signalling molecule is an immunoreceptor tyrosine-based inhibitory motif (ITIM).

16. A nucleic acid molecule according to any one of the preceding claims which additionally encodes one or more secondary signalling motifs.

17. A nucleic acid molecule according to claim 16, wherein each secondary signalling motif is encoded by a SB and is connected to another SB encoding either a primary or secondary signalling motif by a linker containing six or more base pairs.

18. A nucleic acid molecule according to claim 17, wherein each linker encodes the sequence Gly-Ser.

19. A nucleic acid molecule according to any one of claims 16-18, wherein each secondary signalling motif is selected from all or part of CD2, CD4, CD8, CD28 or CD154.

20. A nucleic acid according to claim 19 wherein each secondary signalling motif is selected from all or part of CD28.

21. A nucleic acid molecule according to claim 19 or claim 20, wherein each secondary signalling motif is selected from SB28 or SB29.

22. A nucleic acid molecule according to any one of claims 19-21, wherein said secondary signalling motif is positioned N terminal to said primary signalling motifs.

23. A nucleic acid molecule encoding any one of the synthetic signalling regions listed in Table 1.

24. A nucleic acid molecule according to claim 23, which encodes SB28-SB4*-SB2-SB4.

25. A nucleic acid molecule according to claim 23, which encodes SB28-SB1-SB4".

26. A peptide or polypeptide comprising a synthetic signalling molecule encoded by a nucleic acid molecule according to any one of claims 1-25.

27. A chimeric receptor molecule containing one or more synthetic signalling regions encoded by a nucleic acid molecule according to any one of claims 23-25.

28. A method of generating a synthetic signalling sequence comprising combining two or more primary signalling motifs in an order in which they do not naturally occur.

29. A method according to claim 28, wherein said primary signalling motifs are different primary signalling motifs.

30. A method according to claim 28 or claim 29, additionally comprising combining one or more secondary signalling motifs into the synthetic signalling sequence.

31. A method according to claim 28 or claim 29, wherein said synthetic signalling sequence exhibits increased potency over naturally-occurring signalling sequences in either inducing cell killing, or effecting cytokine release in response to antigen challenge when said synthetic signalling sequence is expressed in an effector cell.

32. A composition comprising a chimeric receptor protein according to claim 27 or a nucleic acid molecule according to any one of claims 1-25, in conjunction with a pharmaceutically-acceptable excipient.

33. A chimeric receptor protein according to claim 27, for use in therapy.

34. Use of a chimeric receptor protein according to claim 27 or a nucleic acid molecule according to any one of claims 1 to 25 in the manufacture of a medicament for the treatment or prevention of a disease in humans.

35. A vector comprising a nucleic acid molecule according to any one of claims 1-25.

36. A host cell transformed with a nucleic acid molecule according to any one of claims 1-25, expressing a peptide or polypeptide according to claim 26 or expressing a chimeric receptor molecule according to claim 27.

FIG. 1

SEQUENTIAL SINGLE SEQUENCE ADDITION

```
Vector BamHI

ACG CGT G                                        GA TCC TGA
TGC GCA CCT AG                                      G ACT
 T   R                                                *

          GA TCA ||||||||||||||||G
BclI           T ||||||||||||||||CCT AG    BamHI

                    Annealed Oligos
```

Correct Orientation:

```
ACG CGT GGA TCA ||||||||||||||||GGA TCC TGA
TGC GCA CCT AGT ||||||||||||||||CCT AGG ACT
 T   R   G   S                     G   S   *
```

Wrong Orientation:

```
ACG CGT GGA TCA ||||||||||||||||TGA TCC TGA
TGC GCA CCT AGT ||||||||||||||||ACT AGG ACT
T   R   G   S                     *   S   *
```

MULTIPLE ADDITION

```
BamH I                                          BamH I

   Bcl----Bam

                Bcl----Bam

                                    Bcl----Bam
```

FIG. 2

CLONING CASSETTE FOR CHIMERIC RECEPTOR CONSTRUCTION

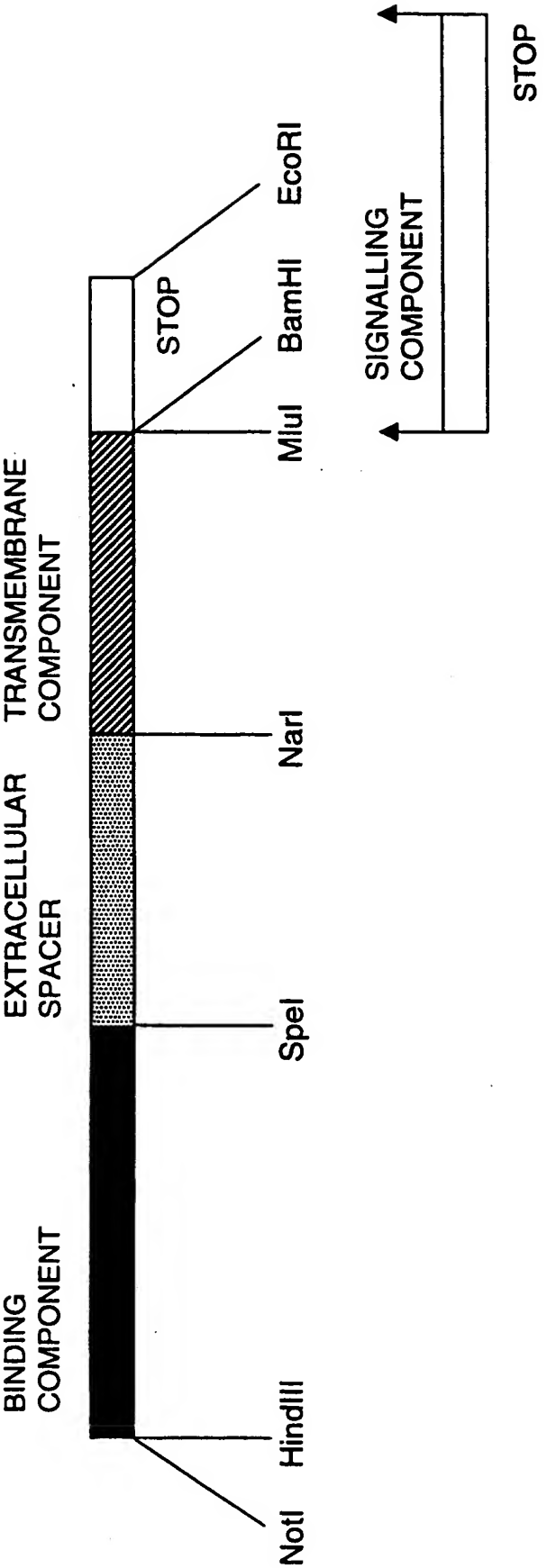


FIG. 3

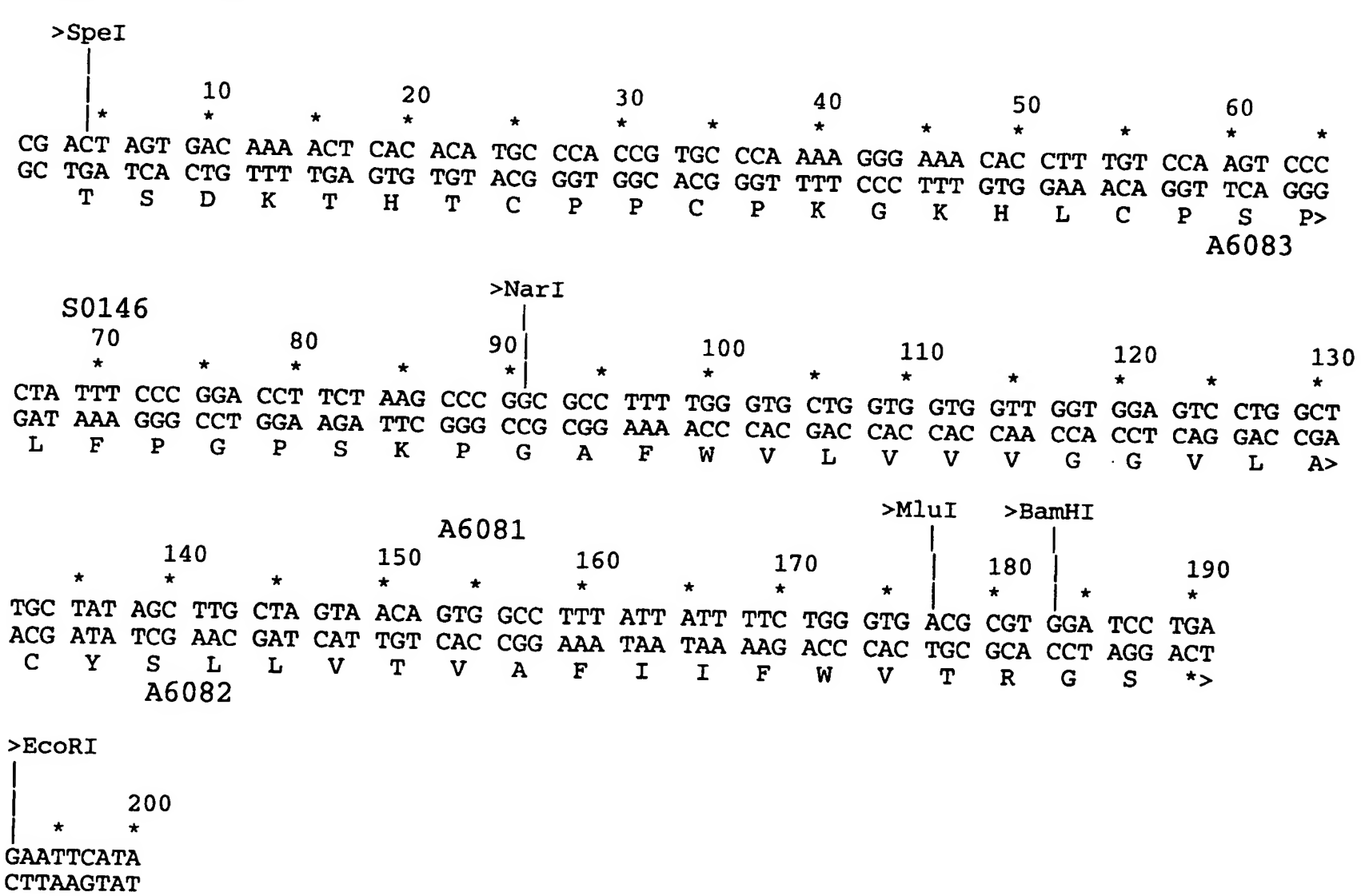

Range: 1 to 200
>SpeI
10
20
30
40
50
60
CG ACT AGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA AAA GGG AAA CAC CTT TGT CCA AGT CCC
GC TGA TCA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT TTT CCC TTT GTG GAA ACA GGT TCA GGG
T S D K T H T C P P C P K G K H L C P S P>
A6083
S0146
>NarI
70
80
90
100
110
120
130
CTA TTT CCC GGA CCT TCT AAG CCC GGC GCC TTT TGG GTG CTG GTG GTG GTT GGT GGA GTC CTG GCT
GAT AAA GGG CCT GGA AGA TTC GGG CCG CGG AAA ACC CAC GAC CAC CAC CAA CCA CCT CAG GAC CGA
L F P G P S K P G A F W V L V V V G G V L A>
A6081
>MluI
>BamHI
140
150
160
170
180
190
TGC TAT AGC TTG CTA GTA ACA GTG GCC TTT ATT ATT TTC TGG GTG ACG CGT GGA TCC TGA
ACG ATA TCG AAC GAT CAT TGT CAC CGG AAA TAA TAA AAG ACC CAC TGC GCA CCT AGG ACT
C Y S L L V T V A F I I F W V T R G S *>
A6082
>EcoRI
200
GAATTCATA
CTTAAGTAT

FIG. 4

OLIGONUCLEOTIDE SEQUENCES FOR CHIMERIC RECEPTOR CONSTRUCTION

All oligos are listed in the 5' to 3' orientation

S0146:CGACTAGTGACAAAACTCACACATGCCCACCGTGCCCAAAAGGGAAACAC
CTTTGTCCAACTCCC

A6081:GCCTTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTT
GCTAGTAACAGTG

A6082:TATGAATTCTCAGGATCCACGCGTCACCCAGAAAATAATAAAGGCCACTGT
TACTAGCAAGCTATAG

A6083:CACCACCAGCACCCAAAAGGCGCCGGGCTTAGAAGGTCCGGGAAATAGG
GGACTTGGAC

A9515:GGCTGATCACGACTGAAGATCCAAGTGCG

A9516:TATGAATTCTCAGGATCCCTGTGGTGGTTTCTCATG

C3208:TATGAATTCTCAGGATCCGCGAGGGGGCAGGGCCTGCATG

C3209:GTGACGCGTGGATCAAGAGTGAAGTTCAGCAGGAGC

D6996:GCCACGCGTGGATCAAGGAGTAAGAGGAGCAGGCTC

D6997:CCGACGCGTGGAGCGATAGGCTGCGAAGTCGCGTGG

A8810:GATCCTGGTTTCTCATGCTTCAGAGTCTCGTAAGTCTCCTGGTTCCTGGTG
CTCAGGCCCGTGTAACACCATCTGATTTCTCATAT

A8810B:GATCCTGGTTTCTCATGCTTCAGAGTATCGTAAGTCTCCTGGTTCCTGGT
GCTCAGGCCCGTGTAACACCATCTGATTTCTCATAT

A8811:GATCATATGAGAAATCAGATGGTGTTTACACGGGCCTGAGCACCAGGAACC
AGGAGACTTACGAGACTCTGAAGCATGAGAAACCAG

A8811B:GATCATATGAGAAATCAGATGGTGTTTACACGGGCCTGAGCACCAGGAAC
CAGGAGACTTACGATACTCTGAAGCATGAGAAACCAG

A8812:GATCCGGCCTGCATGTGAAGGGCGTCGTAGGTGTCCTTGGTGGCTGTACT
GAGACCCTGGTAAAGGCCATCGTGCCCCTGTCCCCTT

A8813:GATCAAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCC
ACCAAGGACACCTACGACGCCCTTCACATGCAGGCCG

A8814:GATCCGCGCTCGCCTTTCATCCCAATCTCACTGTAGGCCTCCGCCATCTTA
TCTTTCTGCAGTTCATTGTACAGGCCTTCCTGAGGGTTCTTCCTT

FIG. 4(CONTD.) 5/17

A8815:GATCAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATA
AGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCG

A8816:GATCCCATCTCAGGGTCCCGGCCACGTCTCTTGTCCAAAACATCGTACTCC
TCTCTTCGTCCTAGATTGAGCTCGTTATAGAGCTGGTTCTGGCCT

A8817:GATCAGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAG
GAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGG

A9000:GATCAGGAAACAAGGTTCCAGAGGATCGTGTTTATGAAGAATTAAACATAT
ATTCAGCTACTTACAGTGAGTTGGAAGACCCAGGGGAAATGTCTCCTG

A9001:GATCCAGGAGACATTTCCCCTGGGTCTTCCAACTCACTGTAAGTAGCTGAA
TATATGTTTAATTCTTCATAAACACGATCCTCTGGAACCTTGTTTCCT

A9002:GATCAAAGCAGACTCTGTTGCCCAATGACCAGCTCTACCAGCCCCTCAAGG
ATCGAGAAGATGACCAGTACAGCCACCTTCAAGGAAACCAGTTGAGGG

A9003:GATCCCCTCAACTGGTTTCCTTGAAGGTGGCTGTACTGGTCATCTTCTCGA
TCCTTGAGGGGCTGGTAGAGCTGGTCATTGGGCAACAGAGTCTGCTTT

A9004:GATCAGCTCTGTTGAGGAATGACCAGGTCTATCAGCCCCTCCGAGATCGA
GATGATGCTCAGTACAGCCACCTTGGAGGAAACTGGGCTCGGAACAAGG

A9005:GATCCCTTGTTCCGAGCCCAGTTTCCTCCAAGGTGGCTGTACTGAGCATCA
TCTCGATCTCGGAGGGGCTGATAGACCTGGTCATTCCTCAACAGAGCT

A9006:GATCACAAAACAAGGAGAGGCCACCACCTGTTCCCAACCCAGACTATGAG
CCCATCCGGAAAGGCCAGCGGGACCTGTATTCTGGCCTGAATCAGAGACGCATC
G

A9007:GATCCGATGCGTCTCTGATTCAGGCCAGAATACAGGTCCCGCTGGCCTTTC
CGGATGGGCTCATAGTCTGGGTTGGGAACAGGTGGTGGCCTCTCCTTGTTTTGT

A9008:GATCACACGTGGATAACGAATACAGCCAACCTCCCAGGAACTCCCGCCTGT
CAGCTTATCCAGCTCTGGAAGGGGTTCTGCATCGCTCCG

A9009:GATCCGGAGCGATGCAGAACCCCTTCCAGAGCTGGATAAGCTGACAGGCG
GGAGTTCCTGGGAGGTTGGCTGTATTCGTTATCCACGTGT

A9010:GATCACCTCCCCGGACCTGCGATGACACGGTCACTTATTCAGCATTGCACA
AGCGCCAAGTGGGCGACTATGAGAACGTCATTCCAGATTTTCCAGAAGATGAGG

A9011:GATCCCTCATCTTCTGGAAAATCTGGAATGACGTTCTCATAGTCGCCCACTT
GGCGCTTGTGCAATGCTGAATAAGTGACCGTGTCATCGCAGGTCCGGGGAGGT

A9012:GATCAGAATATGAAGATGAAAACCTTTATGAAGGCCTGAACCTGGACGACT
GCTCCATGTATGAGGACATCTCCCGGGGCCTCCAGGGCACCTACCAGGATGTGG

A9013:GATCCCACATCCTGGTAGGTGCCCTGGAGGCCCCGGGAGATGTCCTCATA
CATGGAGCAGTCGTCCAGGTTCAGGCCTTCATAAAGGTTTTCATCTTCATATTCT

A9014:GATCAAAGGCTGGCATGGAGGAAGATCACACCTACGAGGGCCTGGACATT
GACCAGACAGCCACCTATGAGGACATAGTGACGCTGCGGACAGGGGAAGTGG

A9015:GATCCCACTTCCCCTGTCCGCAGCGTCACTATGTCCTCATAGGTGGCTGTC
TGGTCAATGTCCAGGCCCTCGTAGGTGTGATCTTCCTCCATGCCAGCCTTT

A9016:GATCACCCCTACCCAACCCCAGGACAGCAGCTTCCATCTATGAGGAATTGC
TAAAACATGACACAAACATTTACTGCCGGATGGACCACAAAGCAGAAGTGGCTG

A9017:GATCCAGCCACTTCTGCTTTGTGGTCCATCCGGCAGTAAATGTTTGTGTCA
TGTTTTAGCAATTCCTCATAGATGGAAGCTGCTGTCCTGGGGTTGGGTAGGGGT

A9018:GATCAAGGCTCCTGCACAGTGACTACATGAACATGACTCCTCGCCGACCAG
GGCCAACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAG

A9019:GATCCTGCGAAGTCGCGTGGTGGGGCATAGGGCTGGTAATGCTTGCGGGT
TGGCCCTGGTCGGCGAGGAGTCATGTTCATGTAGTCACTGTGCAGGAGCCTT

A9020:GATCAATGATCGAAACATACAACCAAACTTCTCCCCGATCTGCGGCCACTG
GACTGCCCATCAGCATGAAAG

A9021:GATCCTTTCATGCTGATGGGCAGTCCAGTGGCCGCAGATCGGGGAGAAGT
TTGGTTGTATGTTTCGATCATT

FIG. 4(CONTD.)

FIG. 5

THE AMINO ACID SEQUENCE OF SEQUENCE BLOCKS

The single letter amino acid code is used

SB1	GSGQNQL**YNEL**NLGRREE**YDVL**DKRRGRDPEMGS
SB2	GSRKNPQEGL**YNEL**QKDKMAEA**YSEI**GMKGERGS
SB3	GSRGKGHDGL**YQGL**STATKDT**YDAL**HMQAGS
SB4	GSYEKSDGV**YTGL**STRNQET**YETL**KHEKPGS
SB4*	GSYEKSDGV**YTGL**STRNQET**YDTL**KHEKPGS
SB5	GSGNKVPEDRV**YEEL**NIYSAT**YSEL**EDPGEMSPGS
SB6	GSKQTLLPNDQL**YQPL**KDREDDQ**YSHL**QGNQLRGS
SB7	GSALLRNDQV**YQPL**RDRDDAQ**YSHL**GGNWARNKGS
SB8	GSQNKERPPPVPNPD**YEPI**RKGQRDL**YSGL**NQRRIGS
SB9	GSHVDNE**YSQP**PRNSRLSA**YPAL**EGVLHRSGS
SB10	GSPPRTCDDTVT**YSAL**HKRQVGD**YENV**IPDFPEDEGS
SB11	GSEYEDENL**YEGL**NLDDCSM**YEDI**SRGLQGTYQDVGS
SB12	GSKAGMEEDHT**YEGL**DIDQTAT**YEDI**VTLRTGEVGS
SB13	GSPLPNPRTAASI**YEEL**LKHDTNI**YCRM**DHKAEVAGS
SB28	GSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAGS
SB29	GSMIETYNQTSPRSAATGLPISMKGS

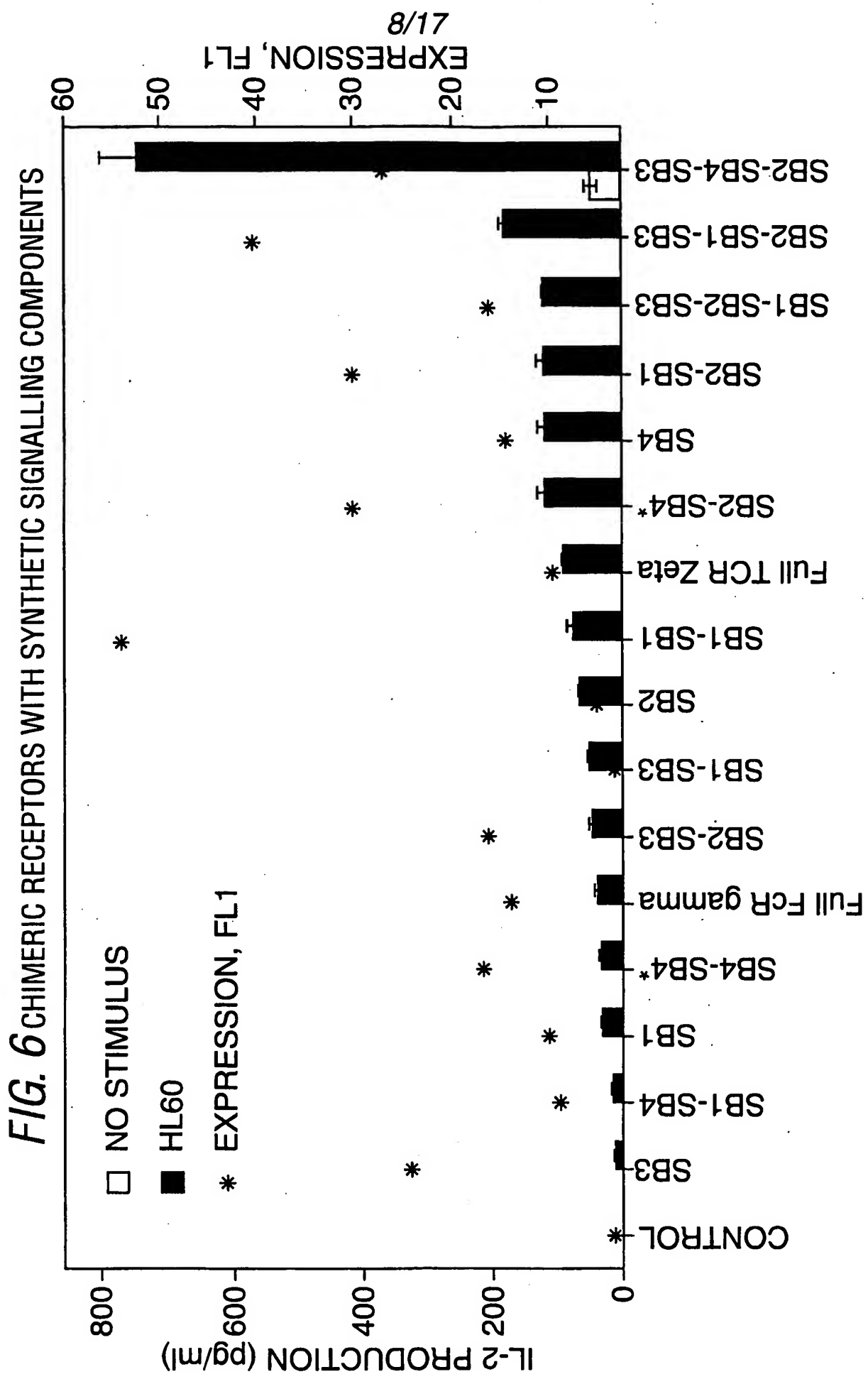


FIG. 6 CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING COMPONENTS

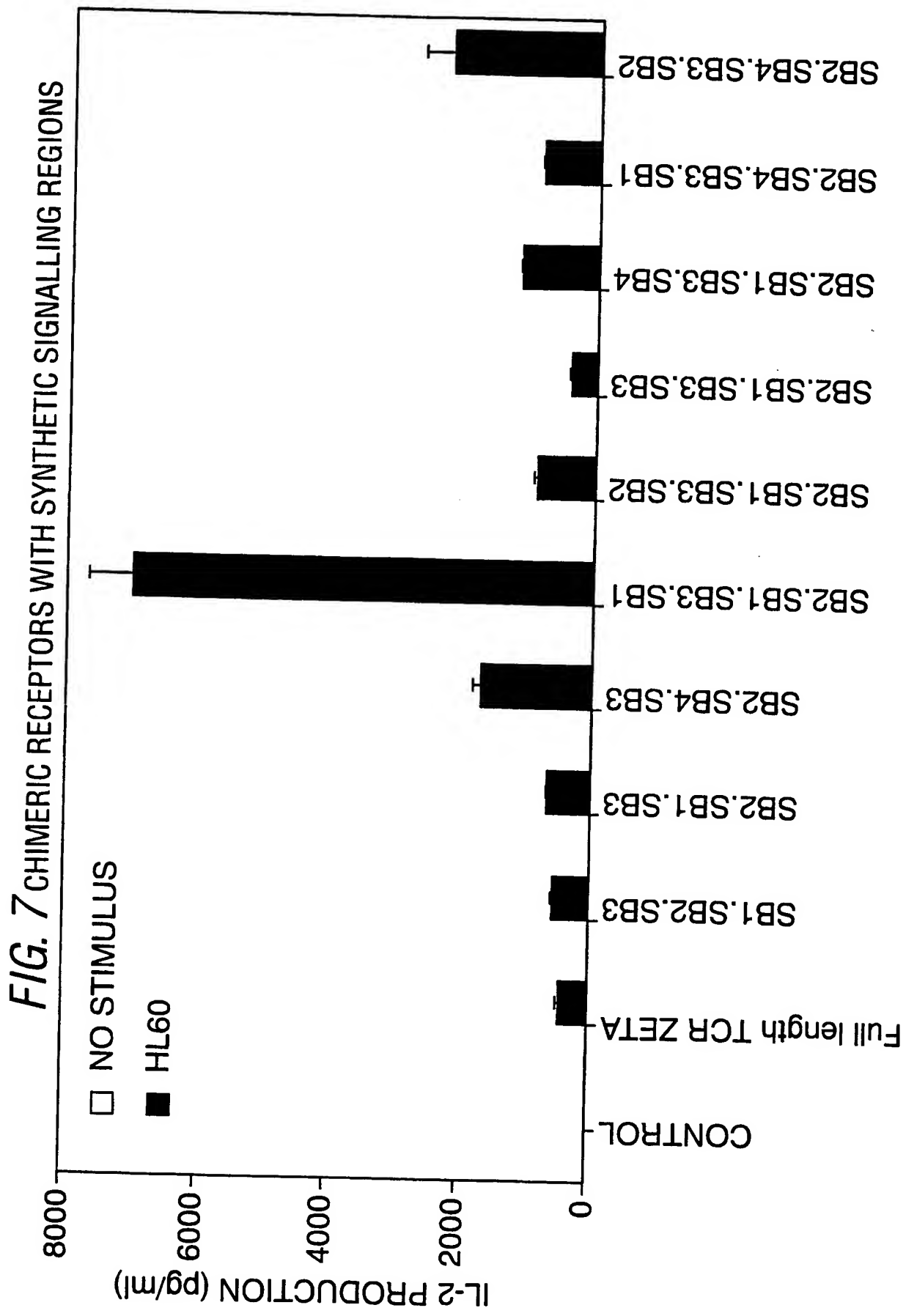


FIG. 7 CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING REGIONS

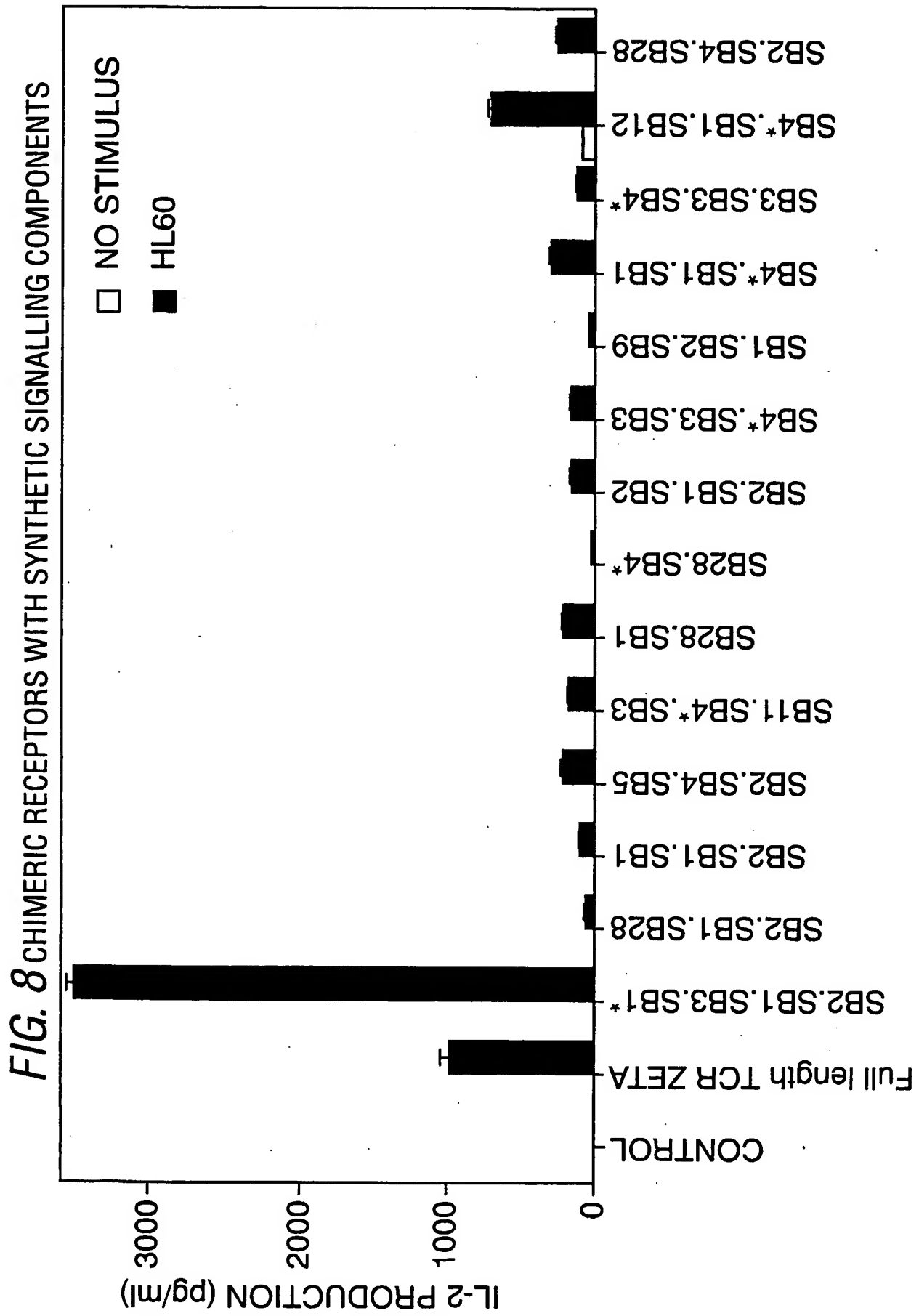


FIG. 8 CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING COMPONENTS

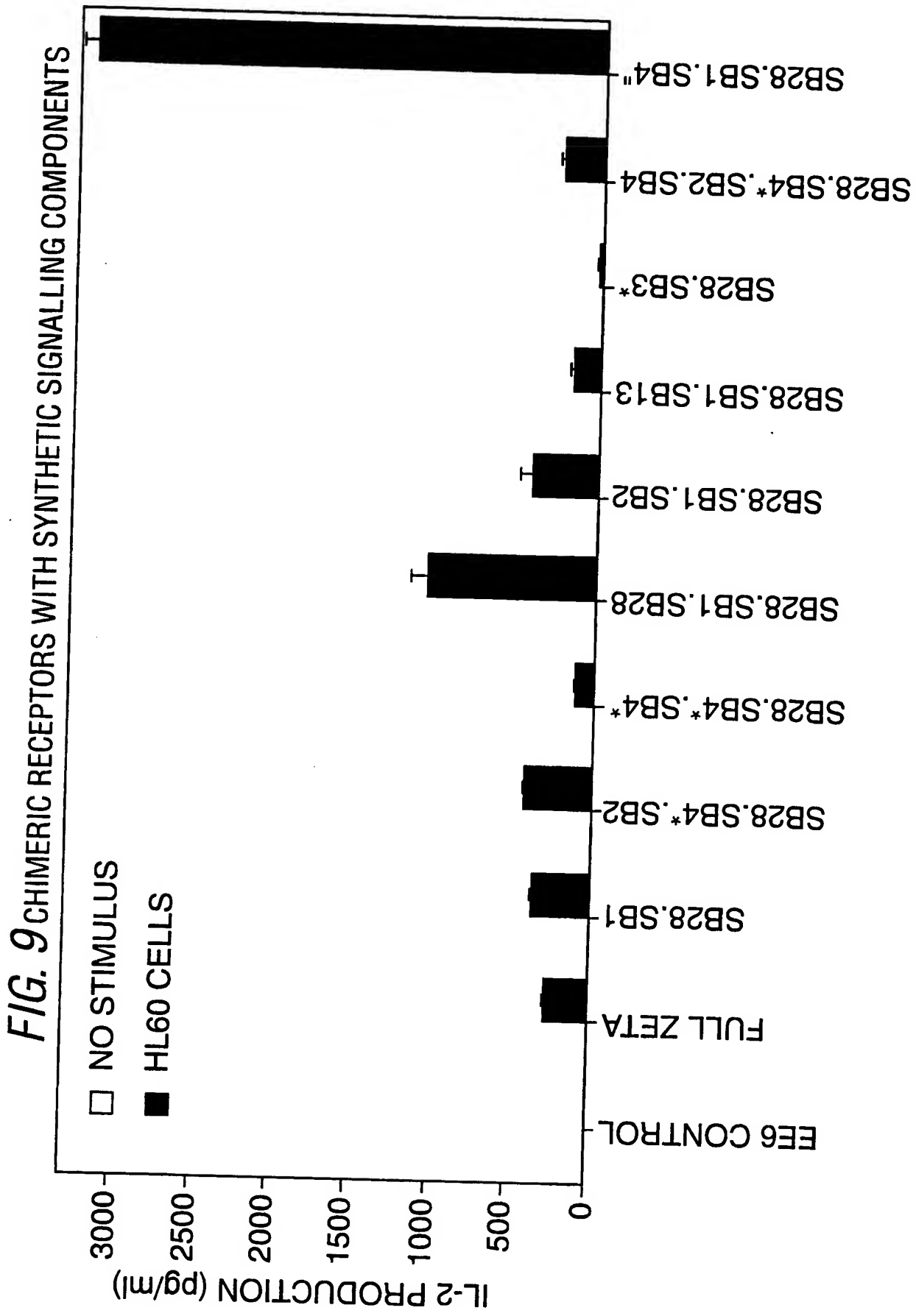
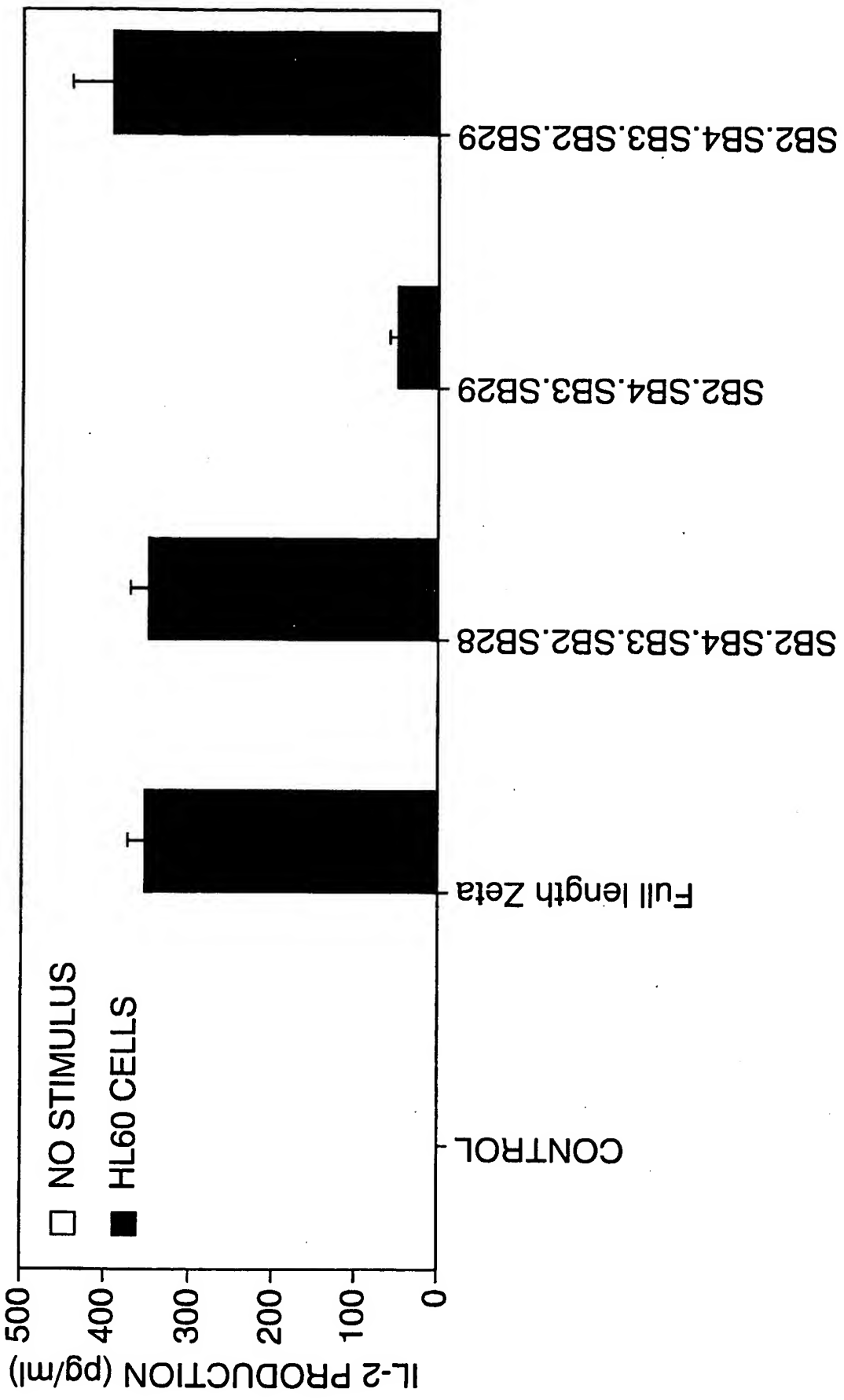


FIG. 9 CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING COMPONENTS

FIG. 10 CHIMERIC RECEPTORS WITH SYTHENTIC SIGNALLING COMPONENTS



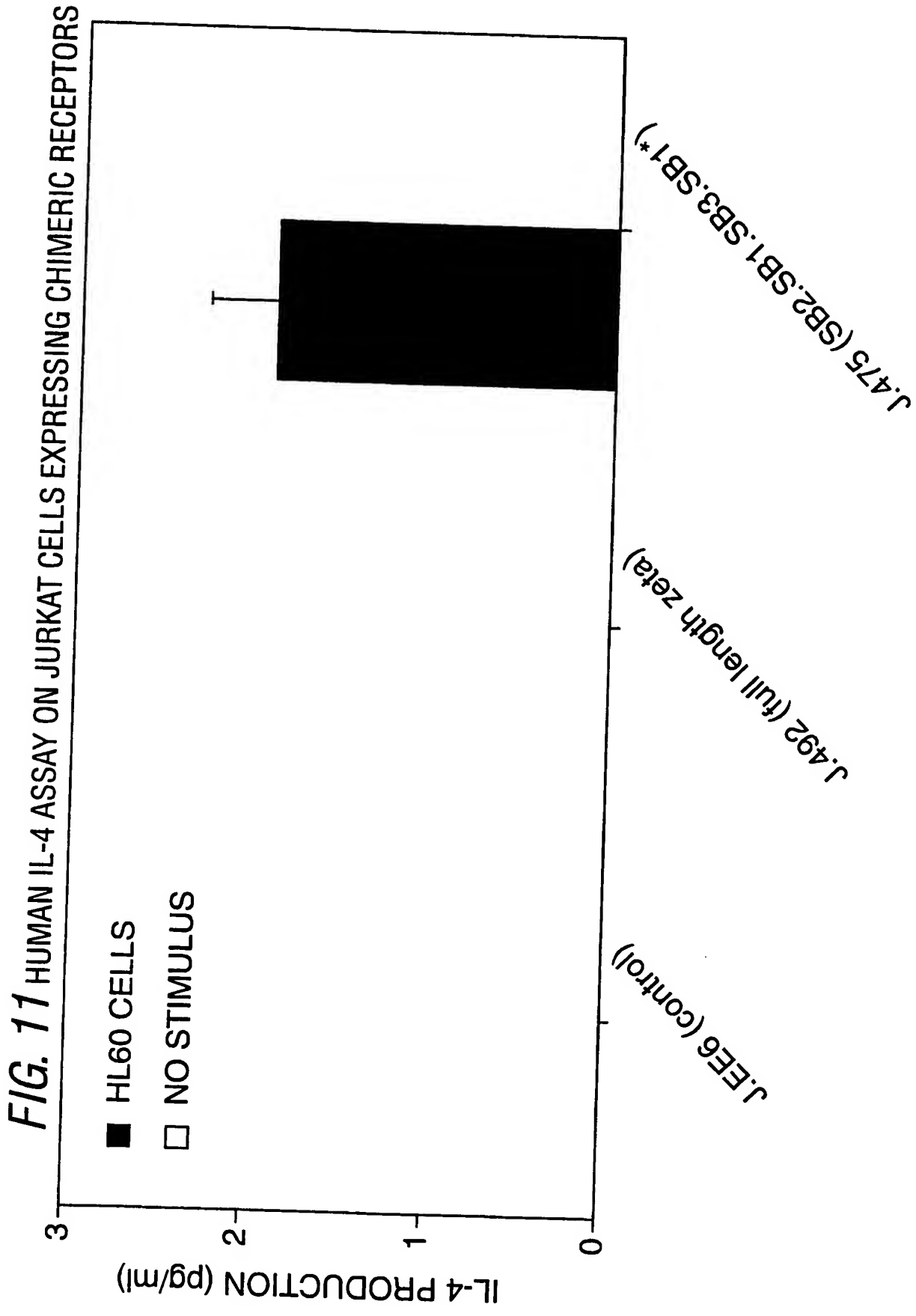
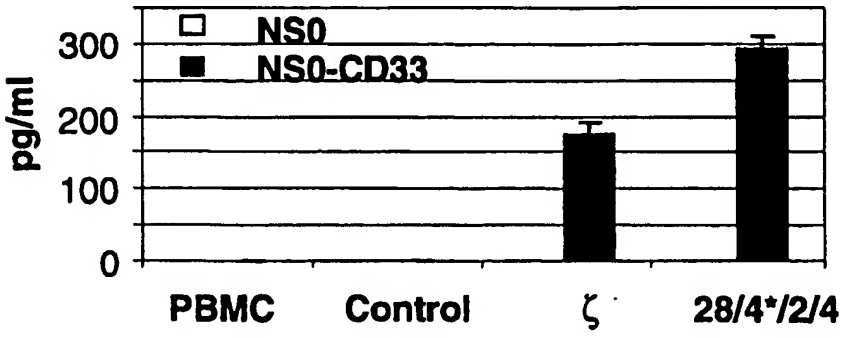
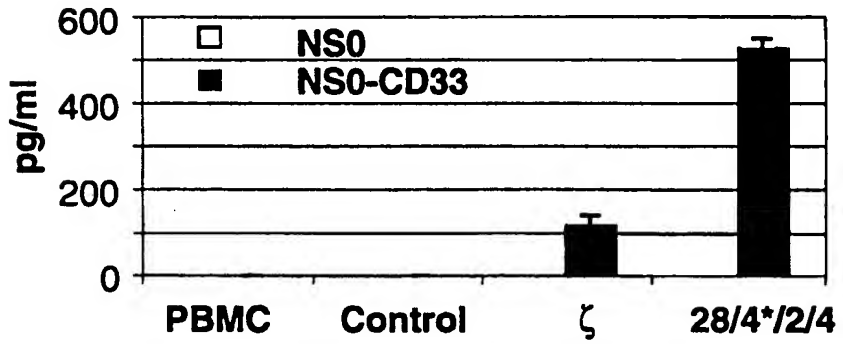


FIG. 11 HUMAN IL-4 ASSAY ON JURKAT CELLS EXPRESSING CHIMERIC RECEPTORS

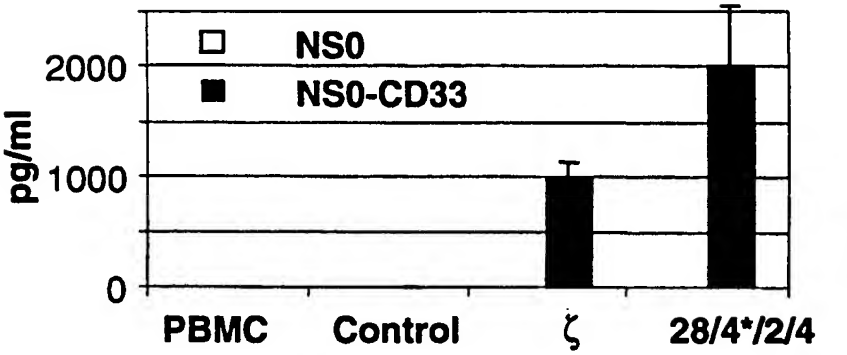
FIG. 12 CYTOKINE PRODUCTION BY HUMAN PBMC EFFECTORS EXPRESSING CHIMERIC RECEPTORS



TNF-α



TNF-β



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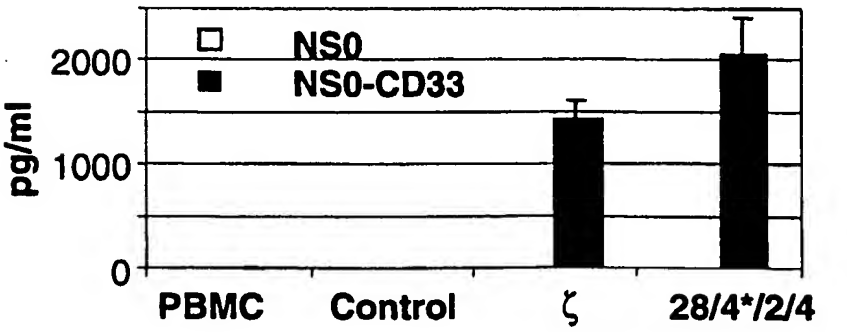
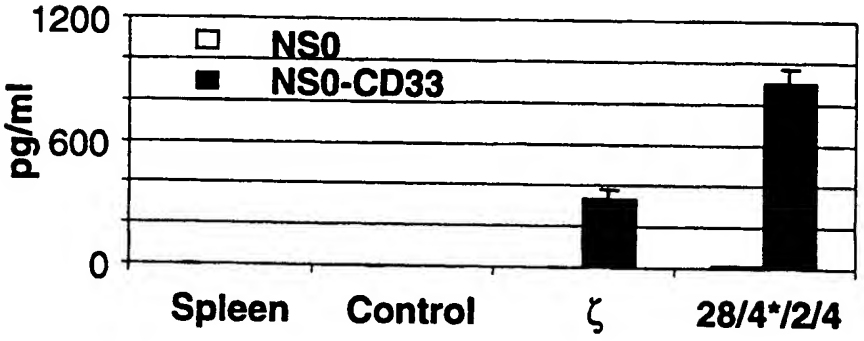
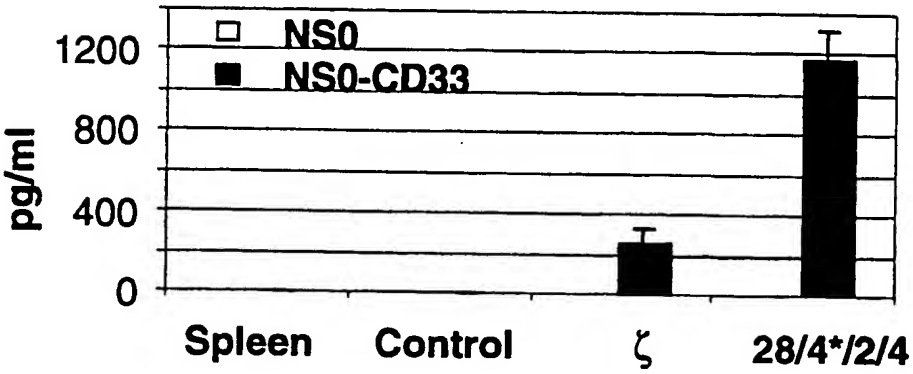


GM-CSF

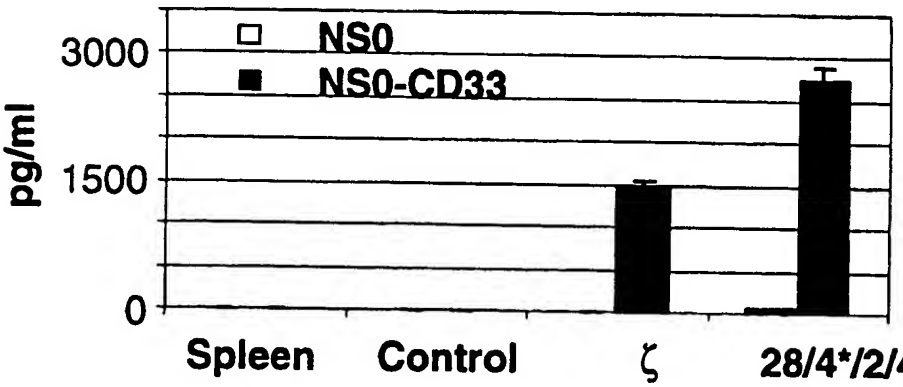
FIG. 13 CYTOKINE PRODUCTION BY MURINE EFFECTORS EXPRESSING CHIMERIC RECEPTORS



TNF-α



IL-2



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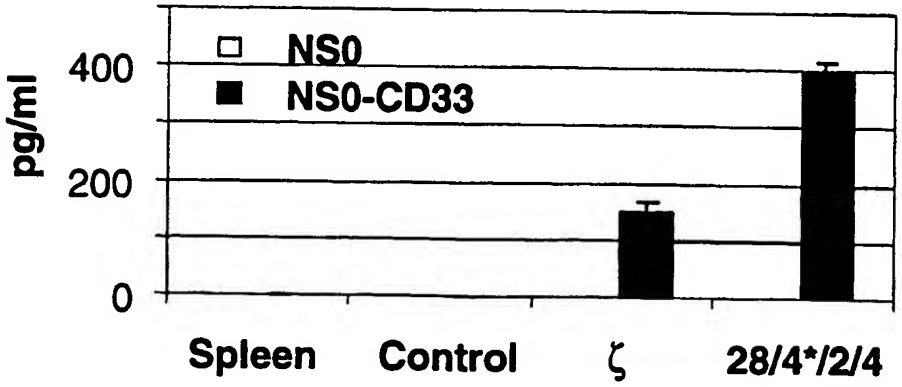


IL-6

FIG. 14 *In-vitro* Cell Killing

Mouse spleen effector cells expressing chimeric receptors
NS0-CD33 target cells

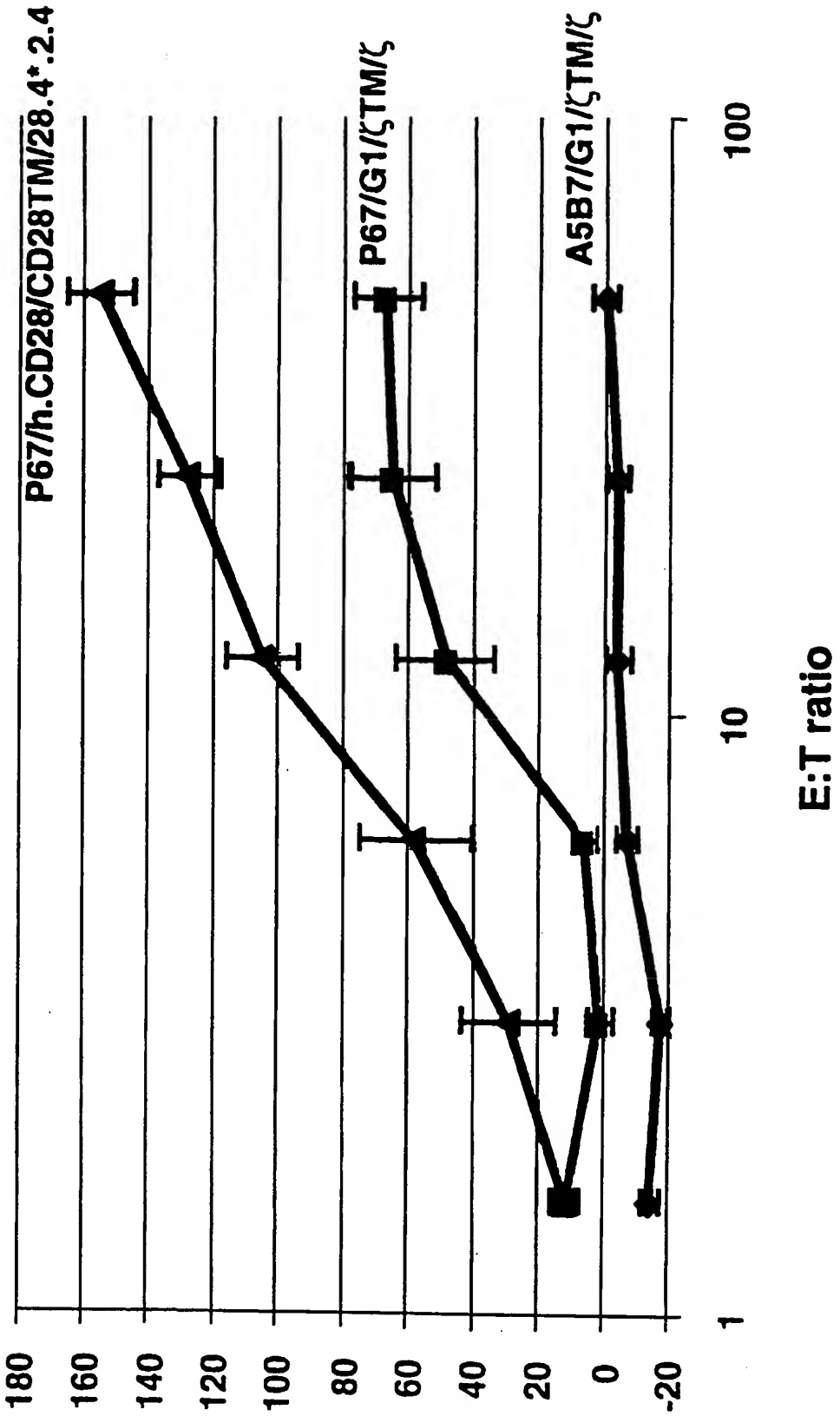
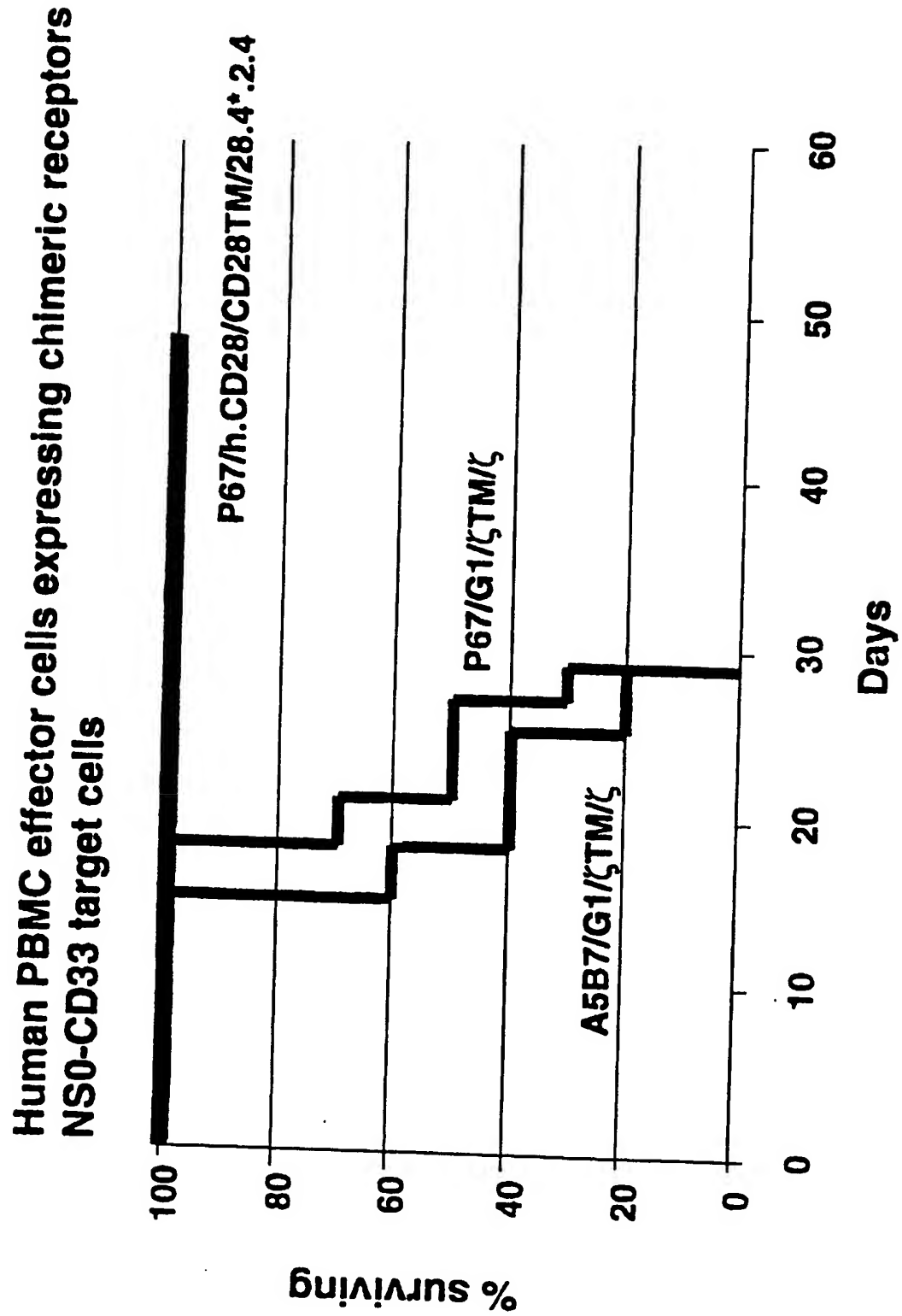


FIG. 15 In-vivo Efficacy 1:1 Premix



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Gly Ser

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Asp Lys Met Ala Glu Ala Tyr Ser Glu Ile Gly Met Lys Gly Glu Arg
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Gly Ser

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Ala Thr Lys Asp Thr Tyr Asp Ala Leu His Met Gln Ala Gly Ser
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Arg Gly Ser
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Lys Gly Ser
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Arg Arg Ile Gly Ser
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Glu Asp Glu Gly Ser
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Phe Ala Gly Ser
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Thr Gly Leu Pro Ile Ser Met Lys Gly Ser
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/705 C12N15/62 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

INSPEC, EPO-Internal, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 23613 A (CELLTECH THERAPEUTICS LTD ;BEBBINGTON CHRISTOPHER ROBERT (GB); LAW) 3 July 1997 (1997-07-03) cited in the application claims 26-28; tables 1,2	1-3,5
X	WO 99 00494 A (CELLTECH THERAPEUTICS LTD ;FINNEY HELENE MARGARET (GB); LAWSON ALA) 7 January 1999 (1999-01-07) cited in the application page 10; example 1	1-3,5
A	WO 96 25953 A (GEN HOSPITAL CORP) 29 August 1996 (1996-08-29) figure 11A	12,26

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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14 August 2000	21/08/2000

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 94 17095 A (ARIAD PHARMA INC) 4 August 1994 (1994-08-04) SEQ ID NOs 5,6,9,11,26,27,29,30	12,26

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